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27. A Simple Method for the Clinical Estimation of Ascorbic Acid in Whole Blood

By **James Deeny, Eric T. Murdock** and **John J. Rogan,** *Lurgan*

[*Received 6 March 1942*]

The procedures suggested for the determination of ascorbic acid in whole blood [e.g. Emmerie & van Eekelen, 1934; Butler & Cushman, 1940; Keuther & Roe, 1941] are generally complicated or require apparatus which is not commonly available. In the course of work on ascorbic acid deficiency we have devised a technique which we have found suitable for clinical use. It is a modification of the method of Butler & Cushman [1940] and is based on the fact that saturation of whole blood with CO prevents the oxidation of ascorbic acid when blood is haemolysed [Kellie & Zilva, 1935; Butler & Cushman, 1939; Fujita *et al.* 1939].

Method

Reagents. (1) 2:6-Dichlorophenolindophenol. Make up a solution in freshly boiled and cooled glass-distilled water of such strength that 1 ml. is equivalent to 0·01 mg. ascorbic acid. This will keep 18–24 hr. without appreciable loss of strength, if in a dark container. (2) Metaphosphoric acid, 32 % solution in glass-distilled water. At room temperature this solution does not keep longer than 36 hr. (3) 2*N* sodium acetate (CH_3COONa, $3H_2O$). (4) Octyl alcohol.

Procedure. Place 2 ml. oxalated whole blood in a test-tube of 2 cm. internal diameter and add 2 drops octyl alcohol. Bubble coal gas through blood for 10 min., add 4 ml. glass-distilled water, continue passage of gas for 5 min. more and then add 2 ml. 32 % metaphosphoric acid and mix by gassing for 30 sec. Add 2 ml. sodium acetate solution and mix by tilting the tube. Transfer to a centrifuge tube and centrifuge. Titrate 2 ml. portions of the supernatant fluid, which should be crystal clear, in the centrifuge tubes, using a standard white light and white background. The indicator is measured from a 2 ml. microburette. A blank consisting of a 2 ml. sample of a mixture of 6 ml. water, 2 ml. metaphosphoric acid and 2 ml. sodium acetate solution, is titrated to a faint pink colour at the same time as the unknown; this facilitates the reading of the end-point of the unknown.

Calculation. If X = titration figure for unknown and Y = titration figure for blank, $\frac{5\,(X-Y)}{2}$ = mg. ascorbic acid/100 ml. blood.

Notes on method

Estimations should be carried out as soon as possible after the withdrawal of blood and care must be taken to avoid haemolysis. Test-tubes of 2 cm. internal diameter are advised because in smaller ones some bloods froth too much and in larger ones oxidation occurs, which can be detected by the alteration in colour of the precipitate from a bright red to a brownish tinge. In passing the gas through blood we have employed a 'wash-bottle' arrangement, using quill tubing. The issuing gas may be burnt. Since some of the sulphur compounds which occur as impurities in coal gas may reduce the indicator, it is advisable to test the gas for H_2S by means of lead acetate paper; if a positive reaction is found the gas should be passed through a KOH solution before it is used.

The accuracy of the method is illustrated by the results of two series of estimations recorded in Tables 1 and 2. Table 1 shows 'recovery' results obtained when ascorbic acid was added to samples of blood of determined ascorbic acid content. Table 2 shows the relative concentrations in plasma and cells and compares the sum of these values with

Table 1. *Recoveries of ascorbic acid added to whole blood*

Sample no.	Ascorbic acid originally present mg./100 ml.	Added mg./100 ml.	Amount found mg./100 ml.	Calculated amount mg./100 ml.	Error %
1	0·83	0·91	1·79	1·74	2·9
2	0·93	0·94	1·84	1·87	−1·6
3	0·93	1·42	2·39	2·35	1·7
4	0·77	0·44	1·20	1·21	−0·8
5	0·20	0·48	0·63	0·68	−7·4
6	0·20	0·97	1·14	1·17	−2·6
7	0·15	0·95	1·14	1·10	3·6
8	0·86	0·93	1·76	1·79	−1·7
9	0·47	0·97	1·45	1·44	0·7
10	0·25	0·75	1·03	1·00	3·0
11	0·29	1·07	1·37	1·36	0·7
12	1·18	0·94	2·12	2·12	0·0
13	0·70	1·02	1·65	1·72	−4·1
14	0·55	1·06	1·62	1·61	0·6
15	0·27	0·96	1·19	1·23	−3·3
16	1·11	0·69	1·73	1·80	−3·9
17	0·92	1·02	1·97	1·94	1·5
18	0·86	0·45	1·37	1·31	4·6
19	0·41	0·91	1·39	1·32	5·3
20	0·93	0·38	1·29	1·31	−2·3

Table 2. *Whole blood determinations checked against plasma and cell concentrations*

Ascorbic acid, mg. found in 100 ml.			Total cell volume/ 100 ml.	Calculated ascorbic acid mg./100 ml. whole blood			Difference between calculated whole blood value and that found by new method %
Whole blood	Plasma	Cells		In plasma	In cells	Total	
0·68	0·44	0·91	42·1	0·26	0·38	0·64	−5·9
0·20	0·01	0·48	40·1	0·00	0·19	0·19	−5·0
0·77	0·47	0·93	57·3	0·20	0·53	0·73	−5·2
0·66	0·48	0·82	41·6	0·28	0·34	0·62	−6·1
1·00	0·63	1·40	46·0	0·34	0·64	0·98	−2·0

those obtained by estimations carried out on the whole blood. Plasma values were determined by a modified form of the method of Farmer & Abt [1936] suggested to us by Dr H. M. Sinclair. The centrifuged cells were made up to their original volume and 2 ml. of the resulting solution were analysed by our method.

Summary

A simple clinical method for the estimation of ascorbic acid in whole blood has been described.

An advantage of the method is that only 2 ml. of blood are required.

We wish to thank the Medical Research Council of Ireland for the grant which made this work possible, Prof. D. C. Harrison (Belfast), Dr L. J. Harris (Cambridge), Dr F. Prescott (The Wellcome Research Institution), and many others who gave their advice and assistance.

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28. The Estimation of the Plasma Proteins by the Salicylsulphonic Acid Reaction

By **Colin Ashley Mawson**, *From the Pathological Laboratory, Royal Berkshire Hospital, Reading*

[*Received 23 February 1942*]

During recent years the increased use of intravenous therapy has led to a development of laboratory control of the more important inorganic constituents of the plasma. It has been customary in this hospital to perform serial estimations of plasma chlorides and alkali reserve in conjunction with frequent determinations of the blood urea when patients have been receiving saline infusions, particularly if duodenal suction, vomiting or copious sweating seemed likely to add to the difficulty of ensuring a correct salt balance. Estimations of the plasma proteins were made from time to time by the micro-Kjeldahl method of King *et al.* [1937], but they were not done as a routine, as the method was not rapid enough to give results at the rate demanded by the large number of samples received. It became evident, however, that a high proportion of the patients had plasma proteins of the order of 4%, below which concentration oedema becomes a serious complication.

Trials were made of several rapid methods for the estimation of plasma proteins and the measurement of the 'optical density' of the cloud produced by the addition of salicylsulphonic acid to a saline dilution of plasma was selected for investigation. Serious objections raised by Plötner [1936, 1] against nephelometric methods based on the use of salicylsulphonic acid have not been found to be applicable to the method adopted, which, though it does not give results comparable in accuracy with the Kjeldahl procedure, is sufficient for clinical purposes and may be applied to serum, plasma, cerebrospinal fluid and urine.

Experimental

Standardization of protein solutions. The protein solutions used in this investigation have all been standardized by a slight modification of the micro-Kjeldahl method of King *et al.* [1937]. It had been found that the porous pot used for the prevention of bumping often formed a fine cloud upon which the Nessler colour was adsorbed. In order to avoid this source of error the digest, after dilution with water, was centrifuged and an aliquot taken for nesslerization. Owing to difficulty in obtaining nitrogen-free reagents, reference curves were constructed for total proteins and non-protein nitrogen by taking nitrogen standards through the complete series of operations.

Apparatus. The 'optical density' of the cloud produced by addition of salicylsulphonic acid to a protein solution was measured in a Klett colorimeter by the photometric method of King *et al.* [1937]. The standard was an Ilford neutral grey screen, and an orange Ilford 'Spectrum' filter was used on the eyepiece of the colorimeter. With the standard screen on the left-hand rack of the colorimeter, the suspension was placed in the micro-cup on the right and the depth of the suspension adjusted until the brightness of the two fields seen in the eyepiece appeared equal. Then

$$\text{'Optical density'} = D_{\text{or.}} = \frac{\text{Density of standard screen}}{\text{Depth (cm.) of suspension}}.$$

The illumination of both sides of the instrument must be equal and the right-hand scale reading must be exactly zero when the cup is racked up into contact with the plunger.

Absorption curve of cloudy suspension. 3 ml. 3% salicylsulphonic acid solution was added to 1 ml. of a 1/100 dilution of plasma in normal physiological saline (0·85% NaCl).

The mixture was shaken and allowed to stand for 10 min. The 'optical density' of the cloudy suspension was determined in the colorimeter, using each of the 8 'Spectrum' filters on the eyepiece. The results are shown in Fig. 1, curve 1. Mr S. Y. Thompson, of

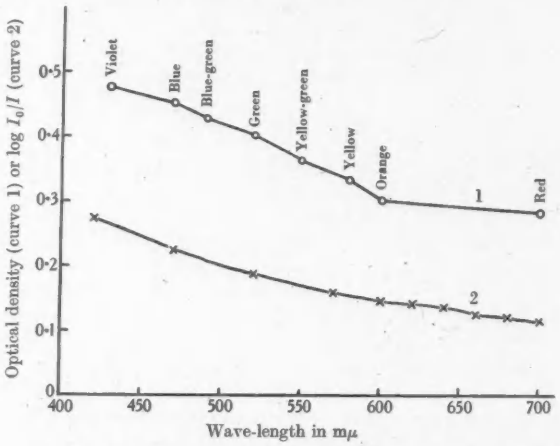

Fig. 1. Absorption curves for salicylsulphonic acid-protein suspensions. Curve 1, using colorimeter and Ilford 'Spectrum' filters; curve 2, using photoelectric spectrophotometer.

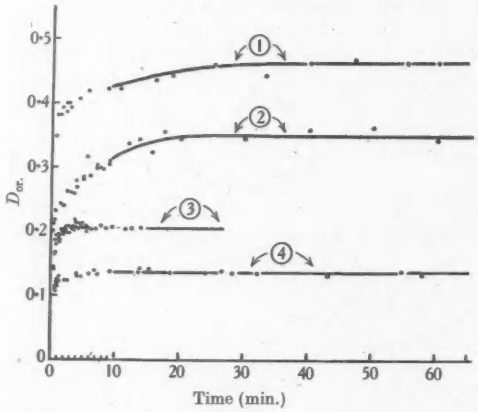

Fig. 2.

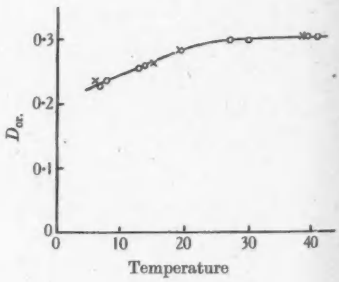

Fig. 3.

Fig. 2. Effect of time on optical density of salicylsulphonic acid-protein suspensions. Curves 1 and 2, plasma albumin; curves 3 and 4, total proteins.

Fig. 3. Influence of temperature on optical density of salicylsulphonic acid-protein suspensions. Time between addition of salicylsulphonic acid and reading of density: circles, 10 min.; crosses, 70 min.

the National Institute for Research in Dairying, Shinfield, kindly determined the extinction coefficients of a similar suspension, using monochromatic light of various wavelengths, in his photoelectric spectrophotometer (Fig. 1, curve 2). The two curves follow one another closely and show a minimum absorption at the red end of the spectrum. As it was desired to eliminate the effect of colour as far as possible, an orange filter was

selected for use on the eyepiece. This colour was preferred to red, as it was found to be less tiring to the eyes when a long series of readings was necessary.

Influence of time. The effect of time on the optical density of the suspension will be seen in Fig. 2. When a 1/100 dilution of plasma in 'normal' saline was used ('Total proteins') the maximum density was attained in 5 min., after which it remained constant. Sedimentation was slow, and though a deposit was present after 24 hr., reconstitution of the suspension by shaking gave a cloud of the original density. This confirms the observation of Plötner [1936, 2].

For the estimation of albumin the Na_2SO_4 filtrate of Howe [1921] was used. 3 ml. 3 % salicylsulphonic acid were added to 0·5 ml. Howe filtrate diluted with 0·5 ml. water. The development of the cloud was slower, but the density remained constant after 30 min. Sedimentation was more rapid, but again reconstitution by shaking was effective.

Influence of temperature. Between 30° and 40° the density attained in 10 min. was independent of the temperature (Fig. 3), but at lower temperatures it was considerably reduced. This was not due to failure to attain maximum density within the time given, as the same result was obtained if the development of the suspension was allowed to proceed for 70 min. The best temperature to select for the reaction would be 38°, but this might introduce certain inconveniences. Laboratory temperatures in England usually range between 17° and 25°, and it will be seen from Fig. 3 that a rise in temperature from 17° to 25° leads to an increase in optical density of about 9 %. It was considered that for most purposes the use of 'laboratory temperature' would be sufficiently accurate, for, if necessary, a correction could be applied from the curve.

Order of addition of reagents. When salicylsulphonic acid was added to a saline dilution of plasma the optical density of the suspension was consistently somewhat higher than when the protein solution was added to the acid. In the case of Howe's albumin filtrate no such consistent difference was observed.

Size of tubes. The reaction was carried out in a series of 10 tubes varying in internal diameter from 2·39 to 0·66 cm. To ensure mixing, the two narrowest tubes (0·80 and 0·66 cm.) had to be reversed twice, while the others were shaken. The same optical density was obtained in all tubes except the two narrowest, in which the results were 8 % higher.

Influence of neutral salts. The presence of 5 % NaCl, Na_2SO_4 or $(NH_4)_2SO_4$ had relatively little effect on the optical density of the suspension, but at higher concentrations a significant reduction occurred. A protein solution giving $D_{or.}$ of 0·370 in normal saline solution gave the following optical densities when salts were added:

	Added salt (%)		
	5	8	11
NaCl	0·361	0·349	0·335
Na_2SO_4	0·349	0·323	0·308
$(NH_4)_2SO_4$	0·341	0·336	0·314

It will be seen from Fig. 4, however, that the cloud produced by globulin is increased by the presence of 11 % Na_2SO_4.

Effect of abnormal albumin/globulin ratio. Since it is not possible to rely upon receiving many samples of plasma having abnormal albumin/globulin ratios in the ordinary course of laboratory practice, a series of mixtures was prepared from several different specimens of plasma or Howe albumin filtrate, to which globulin was added. In this way specimens were obtained with albumin/globulin ratios ranging from 0·16 to 21·6.

The globulin was precipitated from 4 ml. plasma or serum by diluting to 100 ml. with 22·2 % Na_2SO_4. The mixture was kept overnight at 38° and filtered through a 3 G4 Jena sintered glass filter. After being thoroughly drained, the globulin was scraped off the filter and dissolved in 20 ml. normal saline at 38°. The solution was kept at this temperature for 4 hr. and then overnight in the refrigerator, after which it was passed repeatedly

through a 3 G4 filter. A typical product contained 300 mg./100 ml. globulin, 6·0 mg./100 ml. non-protein nitrogen (both by Kjeldahl), and a trace of sulphate.

On addition of salicylsulphonic acid to globulin the density of the suspension was of the same order of magnitude as that obtained with diluted plasma or albumin filtrate, though the straight-line relationship no longer held when the globulin concentration was below 0·1 mg./ml. (Fig. 4). Owing to this fact, addition of very small amounts of globulin in an

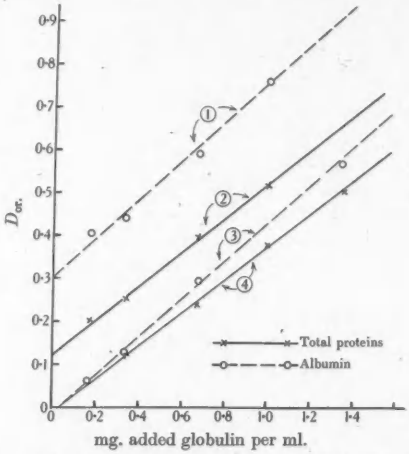

Fig. 4. Addition of globulin to albumin and total plasma protein solutions. Curve 1, addition of globulin in 11·1 % Na_2SO_4 solution to 0·760 mg. albumin; curve 2, addition of globulin in normal saline solution to 0·319 mg. total plasma proteins; curve 3, globulin alone, in 11·1 % Na_2SO_4 solution; curve 4, globulin alone, in normal saline solution.

Table 1. *Addition of globulin to diluted plasma (total protein)*

Protein content = 0·445 mg./ml. Albumin/globulin ratio = 2·18.

Total globulin (mg.)	0·140	0·205	0·269	0·399	0·658	1·012
Added globulin (mg.)	0	0·065	0·129	0·259	0·518	0·962
Total proteins (mg.)	0·445	0·510	0·574	0·704	0·963	1·407
Albumin/globulin ratio	2·18	1·49	1·14	0·765	0·464	0·276
Optical density ($D_{or.}$)	0·202	0·230	0·249	0·317	0·450	0·704
Relative density (mg. total proteins/$D_{or.}$)	2·21	2·22	2·31	2·22	2·12	2·00

Table 2. *Addition of globulin to Howe albumin filtrate*

(*a*) Albumin content 0·908 mg.

Added globulin (mg.)	0	0·042	0·084	0·168	0·252	0·336
Total proteins (mg.)	0·908	0·950	0·992	1·076	1·160	1·244
Albumin/globulin ratio	∞	21·6	10·8	5·39	3·59	2·69
$D_{or.}$	0·428	0·422	0·414	0·469	0·509	0·528
Relative density	2·11	2·25	2·39	2·29	2·48	2·36

(*b*) Albumin content 0·405 mg.

Added globulin (mg.)	0·168	0·336	0·672	1·344	2·016	2·520
Total proteins (mg.)	0·573	0·741	1·077	1·749	2·421	2·925
Albumin/globulin ratio	2·41	1·21	0·601	0·301	0·188	0·161
$D_{or.}$	0·250	0·298	0·454	0·801	1·038	1·275
Relative density	2·29	2·49	2·36	2·18	2·34	2·29

albumin solution may cause no appreciable increase in the density of the cloud obtained with salicylsulphonic acid (Table 2*a*). When, however, globulin is added to a dilution of plasma this 'lag' effect is not seen owing to the fact that the original globulin of the plasma is present.

Fig. 4 shows that the optical density/concentration curve for globulin in saline solution is parallel with that obtained for 'total protein' plus globulin, while the curve for globulin containing 11·1 % Na_2SO_4 is parallel with that obtained for globulin plus Howe albumin filtrate. Tables 1 and 2 illustrate the fact that very large variations in the albumin/globulin ratio cause little alteration in the relative density of the suspension when measured by the photometric technique.

Method

Total plasma proteins. 0·2 ml. plasma was diluted with normal (0·85 %) NaCl to 20 ml. To 1 ml. of this solution were added 3 ml. 3 % salicylsulphonic acid solution. The tube was shaken and allowed to stand for 10 min. It was then shaken again and the optical density of the suspension read in a Klett colorimeter using a neutral grey screen (usually of density 0·50) as standard, with an orange filter on the eyepiece.

$$\text{Optical density} = D_{or.} = \frac{\text{Density of screen}}{\text{Reading of colorimeter (cm.)}};$$

$$\text{Total plasma proteins} = D_{or.} \times 22{\cdot}2\ \%.$$

A standardization curve prepared from numerous samples of plasma is shown in Fig. 5. The lowest point was obtained by using a 1/200 plasma dilution. A similar curve was prepared by Mr Thompson from results obtained with the spectrophotometer, and in this case there was considerably less scatter about the straight line.

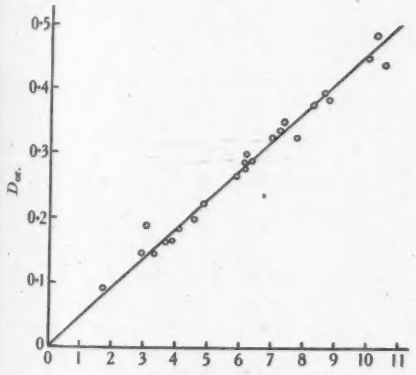

Fig. 5.

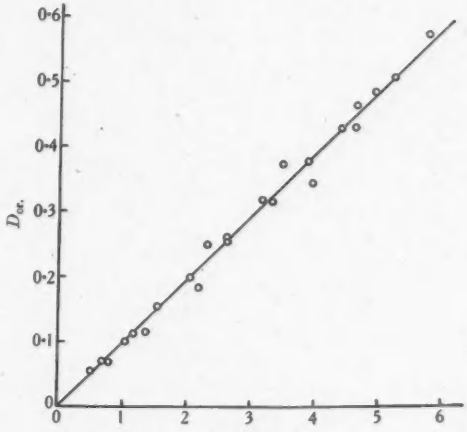

Fig. 6.

Fig. 5. Relation between percentage total plasma proteins and optical density of salicylsulphonic acid-protein suspensions.

Fig. 6. Relation between percentage plasma albumin and optical density of salicylsulphonic acid-protein suspensions.

Albumin. 0·2 ml. plasma was diluted to 5 ml. with 22·2 % Na_2SO_4 solution. After remaining for 3 hr. at 38° the mixture was filtered at 38° through two no. 2 Whatman filter papers. 0·5 ml. filtrate was diluted with 0·5 ml. water, and 3 ml. 3 % salicylsulphonic acid solution were added. The tube was shaken, left to stand for 40 min. and shaken again. The optical density was then read as before:

$$\text{Plasma albumin} = D_{or.} \times 10{\cdot}3\ \%.$$

A standardization curve for albumin, prepared from numerous Howe albumin filtrates, is shown in Fig. 6. In a few cases low results for albumin have been observed with the above procedure. Improved results were obtained by diluting 1 ml. plasma to 25 ml. with 22·2 % Na_2SO_4, the mixture being kept at 38° overnight. Owing to the extra time involved this has not been adopted as a routine.

Total proteins of cerebrospinal fluid or urine. 1 ml. of cerebrospinal fluid or urine (diluted if necessary with normal saline) was used instead of the diluted plasma. If the urine after filtration was cloudy or highly coloured, a blank was run using urine alone in the colorimeter cup. The optical density so obtained was divided by 4 and subtracted from that of the suspension. For this purpose a neutral grey screen of density 0·125 was used as a standard.

$$\text{Total proteins} = D_{\text{or.}} \times 222 \text{ mg./100 ml.}$$

Discussion

When it became necessary to find a rapid micro-method for the serial estimation of proteins in large numbers of specimens of plasma or serum, the biuret method of Robinson & Hogden [1940] was tried with promising results. It is rapid and gives a colour of good optical properties which was measured photometrically, using a yellow-green filter. Results were highly reproducible in the sense that duplicates were as a rule almost identical, but when the method was standardized against the Kjeldahl procedure the variations observed were disappointing.

King & Haslewood [1936] have described a turbidometric method for the estimation of proteins using the turbidity produced by salicylsulphonic acid and comparing this with a series of permanent standards prepared from a suspension of formazin in gelatin. While this procedure was not quite accurate enough for the estimation of plasma proteins, the method has been adapted for use in the colorimeter and this modification has increased the accuracy sufficiently to make it suitable for clinical investigations. The curve obtained (Fig. 5) relating the optical density of the suspension to the protein content found by the micro-Kjeldahl method was a straight line passing through the origin, which compares favourably with the sigmoid curve reported by Looney & Walsh [1939, 1] for the salicylsulphonic acid reaction in presence of gum ghatti.

The latter authors determine globulin by turbidometric estimation of the cloud produced by $(NH_4)_2SO_4$ in presence of gum ghatti [Looney & Walsh, 1939, 2], but although their reference curve is a straight line, it cuts the globulin axis slightly below 1 %. In the present investigation it has been found that the reference curve for globulin solutions plus salicylsulphonic acid also cuts the globulin axis at about 0·4 %. Dulière [1937] has stated that the use of $(NH_4)_2SO_4$ for the estimation of globulin is undesirable, as it does not give the same results as the classical method of Howe which he regards as the standard procedure. It has been found that the Howe albumin filtrate can be used for the estimation of albumin by the salicylsulphonic acid technique, although a slightly different relative optical density is obtained in these circumstances, necessitating the use of a different reference curve for the estimation of albumin (Fig. 6).

The chief difficulty in the way of the use of the salicylsulphonic acid reaction for the estimation of proteins lies in an observation of Plötner [1936, 1] who found that the addition of globulin to an albumin solution led to a *decrease* in the turbidity produced by salicylsulphonic acid, as measured by the nephelometer. This decrease was progressive as more globulin was added, until a minimum density was reached when the albumin/globulin ratio was unity. From this minimum the turbidity increased in proportion to the added globulin, the reference curve becoming parallel with that obtained for globulin solution alone. Plötner also observed that the turbidity produced by globulin alone was only one-fourth of that produced by the same amount of albumin. When Plötner [1936, 2] determined the density of the suspension, per unit weight of protein, in a series of patho-

logical plasmas of widely different albumin/globulin ratio, variations up to 300% were observed. For the method to be valid for clinical use this relative density must be constant for all specimens of plasma, and Plötner therefore concluded that the salicylsulphonic acid method was useless for the estimation of the plasma proteins.

It has been shown in the experimental section that when the salicylsulphonic acid reaction is carried out in the manner described, and the density of the cloud is measured photometrically in a colorimeter, the objections raised by Plötner's observations do not apply. This can be seen in Fig. 4, where it is evident that no tendency exists to form a minimum in the optical density/added globulin curve at the point at which the concentrations of albumin and globulin are equal. This is confirmed in Tables 1 and 2, where fairly constant figures are shown for the relative density of the cloud over a very wide range of albumin/globulin ratios. It must be admitted that the globulin used was not pure, but it was of about the same quality as that used by Plötner, and the density of the suspension formed by globulin alone was of the same order of magnitude as that due to albumin or to diluted plasma of the same concentration.

Table 3. *Estimation of proteins by the salicylsulphonic acid method; effect of variation of albumin/globulin ratio*

Patient	Diagnosis	Total proteins %		Albumin %		Albumin/globulin ratio	
		Kj.	S.S.A.	Kj.	S.S.A.	Kj.	S.S.A.
Br (1)	Eosinophilia and anaemia	9·9	9·9	1·5	0·5	0·18	0·05
(2)	of unknown origin;	8·8	8·4	1·4	1·0	0·19	0·13
(3)	(? trichiniasis)	12·0	10·9	1·8	1·0	0·18	0·10
Co (1)	Nephrosis with lipaemia	3·7	3·6	0·7	0·7	0·23	0·26
(2)		3·9	3·7	0·8	0·7	0·25	0·24
Ha	Multiple myelomatosis	10·5	9·7	2·2	2·0	0·26	0·25
Ga	Chronic nephritis	4·6	4·4	1·4	1·2	0·43	0·42
Gr	Nephrosis with lipaemia	2·9	3·2	1·2	0·4	0·66	0·13
Mo	Acute retention (E.P.)	6·1	6·0	3·3	3·3	1·2	1·2
Os	Acute retention (E.P.)	5·9	5·9	3·2	3·4	1·2	1·3
Do	(Stored plasma)	4·8	4·9	2·6	2·7	1·2	1·2
To	(Stored plasma)	6·4	6·2	4·0	3·7	1·7	1·4
St	Chronic retention	6·1	6·3	4·0	3·7	1·9	1·4
Ga	Anastomotic ulcer	7·0	7·2	4·9	5·2	2·4	2·6
Cl	Pyloric stenosis	7·2	7·4	5·2	5·2	2·7	2·4

Kj. = Kjeldahl method; S.S.A. = salicylsulphonic acid method.

The results of the estimation of total proteins and albumin in 15 specimens of plasma are given in Table 3. The albumin/globulin ratios range from 0·18 to 2·7, and it is evident that discrepancies between the Kjeldahl and salicylsulphonic acid methods for the estimation of total proteins are not correlated with variations in this ratio. However, in two cases ('Br', with 3 different samples taken over a period of one month, and 'Gr') very low results were obtained for plasma albumin by the salicylsulphonic acid method. In both cases the albumin/globulin ratio was low, but the errors occurred in the albumin determination, not in the total proteins, as would be expected from Plötner's results. No explanation for these results has been found, since correct results have been obtained with other plasmas of apparently similar composition.

It must be emphasized that no high degree of accuracy is claimed for the method described, which is designed for the rapid assessment of the protein needs of a patient receiving intravenous therapy, where precise results are not necessary. When exact figures are required the Kjeldahl method should be used. In a series of 29 determinations of total plasma proteins and 24 determinations of plasma albumin the following results were obtained:

	Conversion factor (% protein by Kjeldahl) $D_{or.}$	Standard error	Standard deviation
Total plasma proteins	22·2	±0·22	±1·21
Plasma albumin	10·3	±0·13	±0·64

SUMMARY

1. The suitability of the salicylsulphonic acid reaction for the photometric estimation of total plasma proteins and plasma albumin has been investigated.

2. Methods are described for the use of this reaction for the estimation of the proteins of plasma, cerebrospinal fluid and urine.

The author's thanks are due to Dr E. J. King and Mrs M. E. Boyland for their assistance in the revision of the manuscript, and to Mr S. Y. Thompson for estimations which he performed with the spectrophotometer.

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29. Estimation of Blood Creatinine

By **G. E. Delory** and **J. Jacklin**, *From the British Postgraduate Medical School, London, W. 12*

(*Received 10 March 1942*)

Creatinine is usually estimated by the method of Folin & Wu [1919], which depends on a comparison with a standard of the colour produced when a deproteinized blood filtrate is treated with alkaline picrate. It is well recognized that the accuracy of the method is severely limited by the fact that the colour produced with the normal range of creatinine is not very much greater than that of the alkaline picrate itself.

The purpose of this paper is to show how the colour of the blank may be corrected for by a simple algebraic calculation, considerably greater accuracy being thus attained.

Beer's law, on which colorimetry is based, states that light in passing through a coloured medium is absorbed in direct proportion to the concentration of coloured substance. Thus the concentration of coloured substance is inversely proportional to the observed depth in a Duboscq colorimeter. In the case of creatinine, the estimation cannot be accurately made because of the large blank colour due to the excess of the alkaline picrate reagent used. The following calculation, applied to the colorimetric readings obtained in the usual way, obviates this difficulty. Let C_S, C_T and C_B be the concentrations of the standard, test and blank, and R_S, R_T and R_B be the corresponding colorimetric readings. Then

$$C_S \propto \frac{1}{R_S} - \frac{1}{R_B} \quad \text{and} \quad C_T \propto \frac{1}{R_T} - \frac{1}{R_B},$$

when by division

$$\frac{C_T}{C_S} = \frac{1/R_T - 1/R_B}{1/R_S - 1/R_B}.$$

Hence

$$\frac{C_T}{C_S} = \frac{(R_B - R_T)/R_T R_B}{(R_B - R_S)/R_B R_S}.$$

And

$$C_T = \frac{R_B - R_T}{R_B - R_S} \times \frac{R_S}{R_T} \times C_S.$$

Table 1 compares the results obtained by means of this calculation with those resulting from the usual calculation, i.e. $C_T = R_S/R_T \times C_S$, when solutions of known creatinine content were used. Table 2 gives a comparison for recoveries from whole blood, while Table 3 gives the results obtained on blood samples sent to the laboratory for routine examination.

Table 1. *Colorimetric analysis of standard creatinine solutions*

Stated conc. mg./100 ml.	(1) By usual calc. mg./100 ml.	(2) By proposed calc. mg./100 ml.	Stated conc. mg./100 ml.	(1) By usual calc. mg./100 ml.	(2) By proposed calc. mg./100 ml.
2·5	1·28	2·55	12·5	3·46	12·70
5·0	1·80	5·00	15·0	4·00	15·20
7·5	2·40	7·80	17·5	4·50	17·50
10·0	3·00	10·60	20·0	5·10	20·50

The comparisons were made in a Duboscq colorimeter; matching was facilitated by the use of an Ilford blue-green spectral light filter [King & Haslewood, 1937].

Table 2. *Recovery of creatinine added to blood*

mg./100 ml. added to blood	mg./100 ml. recovered	
	By usual calc.	By proposed calc.
2·5	1·3	2·6
5·0	1·8	5·0
10·0	3·1	9·9
12·5	3·5	12·5

Table 3. *Creatinine concentrations of hospital blood samples by two methods of calculation*

mg./100 ml.		mg./100 ml.	
By usual calc.	By proposed calc.	By usual calc.	By proposed calc.
0·8	0·9	2·0	6·6
1·2	1·2	2·2	6·3
1·3	1·5	2·3	6·5
1·3	1·6	2·6	6·6
1·6	3·3	3·9	14·2

It would of course be unnecessary to correct for the blank if it were possible to have a standard which exactly matched each test, but this is impracticable in a routine method. This point is well illustrated in Table 3. The standard used was equivalent to 1 mg. of creatinine per 100 ml. of blood. When the blood concentration is near this figure the agreement between the results obtained by the two methods of calculation is good. But as the concentration of the test departs from that of the standard, the results obtained by the ordinary calculation become increasingly inaccurate. With the proposed calculation only one standard is needed, and the figures shown in Tables 1 and 2 show an accuracy previously unattained in creatinine estimations. A further advantage of this method is that the tedious purification of the picric acid need not be carried out. It may be objected that since the substance estimated by the picric acid methods may not actually be creatinine, increased accuracy in measurement is pointless; but the chromogenic substance, even if not creatinine, is of considerable importance in clinical biochemistry, and an accurate estimation is extremely useful.

The authors are very grateful to Dr E. J. King for his continual help and encouragement throughout the investigation.

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30. The Spectrocomparator

An Instrument for estimating the Concentration of Pigments in the presence of other Pigments, and for comparing Absorption spectra*

By **Philipp Ellinger**, *From the Lister Institute of Preventive Medicine, London*

(*Received 2 March 1942*)

The idea of the instrument originates from the consideration of two practical problems. During the course of an investigation on the pigments present in pathological urines, an instrument was required to enable an estimation of a particular pigment possessing a characteristic absorption band to be carried out in the presence of other pigments. Secondly, the separation of pigments by means of chromatographic absorption analysis made it desirable to compare immediately the spectra of serial eluates. There appeared to be no simple instrument suitable for this purpose available in this country. From Weigert's [1927] description, however, it seemed that three German firms (Krüss, Zeiss, and Schmidt and Haensch) had manufactured instruments called 'Spectralkolorimeters' which might partly have served the purposes mentioned. Weigert considered these Spectralkolorimeters to be less accurate than the usual absorptiometers†, as the presence of the whole spectrum tends to distract the eye from the band. While the spectrocomparator now to be described was under construction, Perlman [1939] suggested mounting a hand spectroscope immediately above the prisms of a standard Duboscq absorptiometer, with the slit perpendicular to the line of division. It could not be ascertained whether an instrument of this design had ever been made.

The Instrument

The spectrocomparator consists of an ordinary Duboscq absorptiometer with a variable slit attached immediately above the top of the biprism so that the division line and the slit can be focussed simultaneously by the lens of the eyepiece. The ordinary eyepiece of the absorptiometer is replaced by a special eyepiece containing the necessary arrangement for dispersing the two beams of white light passing the biprism and the eye lens.

Illuminating apparatus and instrument are fixed on a board or an optical bench. The illumination is provided by an opalescent 200 W. lamp at a short distance from the mirror in order to give intense illumination. The lamp-holder is adjustable in all directions in order to illuminate the mirror equally. The lamp is covered by a reflecting tin shade which is designed to prevent light from entering the observer's eye but to allow sufficient ventilation for cooling purposes. The general arrangement of the instrument can be seen from Fig. 1 *a* and *b*. Above the mirror *A* two cups *BB* are attached with bottom plates *CC* made of optical glass. They hold the solution to be examined and the standard solution respectively. Fixed plungers of optical glass *DD* dip into these cups, the level of which can be altered by a micrometer screw to regulate the depth of solution. On top of the plungers is a Duboscq prism *E*, and above this the biprism *F*, the sharp edge of which provides the division line of the two fields. Immediately above this biprism is attached an adjustable slit *G*, with its opening perpendicular to the division line. If entirely opened, it does not

* The instrument was demonstrated at the Hampstead meeting of the Physiological Society on 16 December 1939.

† The word 'absorptiometer' should be used exclusively for instruments estimating the concentration of a pigment from its light absorption, while the word 'colorimeter' should be reserved for instruments devised to match the qualities of a colour.

interfere with the aperture of the absorptiometer. The top of the instrument is formed either by the usual eyepiece when the instrument is used for normal absorptiometric purposes, or the eyepiece is replaced by the special eyepiece shown in Fig. 1. It consists of an eye lens *H* which allows simultaneous focussing of the slit and division line of the biprism. On top of its holder rests a small-angle prism *I*, the top surface of which is inclined by 45°. It provides some dispersion and straightens the beam. On its upper surface a celluloid replica of a grating *J* is fixed. This grating provides the actual dispersion. Above the prism *I* a ring-shaped filter-holder *K* is attached, and on this can rest small circular filters *L*. The eyepiece is terminated at the top by an oblong diaphragm *M*, with its length parallel to the division line, providing even illumination of both spectra and preventing disturbing reflexions. At the level of the top surface of the prism *I* the eyepiece tube carries a side tube. This side tube bears on its free end an opalescent cover-glass *N*, behind that a negative scale *O*, and beyond the scale a collector lens *P* projecting an image of the scale on the surface of the prism, which reflects this image through the diaphragm to the eye. The scale is illuminated by a 3·5 V. lamp *Q* fixed in a tube which can be plugged on the far end of the side tube.

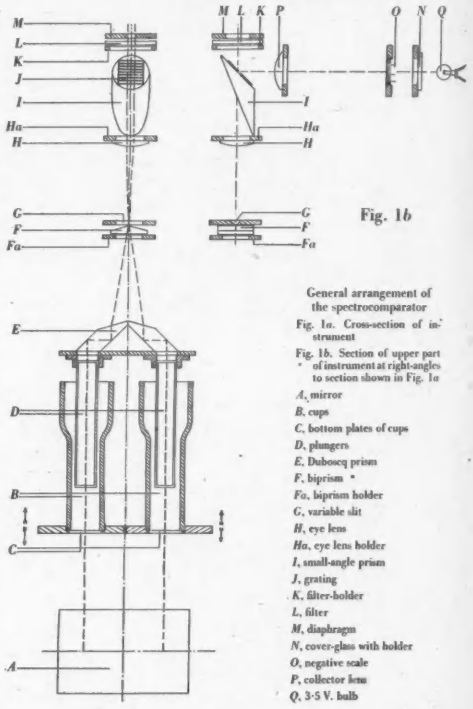

Fig. 1a

The two beams (indicated by dotted lines) passing through the solutions and plungers into the Duboscq prism are twice reflected into the refracting biprism where the beams are crossed. They next pass through the slit and the eye-lens into the prism and the grating. Here the light is dispersed and two parallel spectra are formed, separated by an almost invisible division line. The spectra are observed through the diaphragm. By interposing filters transmitting various portions of the spectrum small zones of the spectrum can be separated for observation. By the side of the spectrum the image of the scale appears.

The instrument was designed but not completed before the war. Owing to the emergency it had to be completed in a way that involved as little labour as possible, and therefore it has not been possible to incorporate certain features that would be of considerable value. Improvements are possible in two directions. First, the eyepiece should be attached to the instrument obliquely to the main tube so that the reading can be done with the head in an unstrained position; secondly, the dispersion should be done exclusively by means of a grating, providing even dispersion over the whole range of the spectrum and finally, the determination of wave-lengths should be carried out by shifting a pair of cross-wires along the spectrum by means of a micrometer screw. The two cross-wires should be fixed vertically one above the other to avoid parallax in reading. A drum fitted to the micrometer screw could be calibrated in wave-lengths.

The use of the instrument

The instrument can be used for the following purposes:

(1) As an ordinary absorptiometer, by opening the whole aperture of the slit and using the ordinary absorptiometer eyepiece.

(2) As a very bright spectroscope, by using the spectral eyepiece, closing the slit to a small opening and using only one of the cups. This spectroscope has the great advantage that the depth of the layer of the solution to be examined can be changed without difficulty and accurately measured. The instrument is superior to and easier to handle than the Bailey tube or similar arrangements.

(3) For the estimation of the concentration of a pigment in the presence of other pigments. This can be done only if the pigment to be examined has an absorption band or line in a region of the spectrum where the other pigments do not absorb. For this purpose one cup is filled with a pure solution of known concentration of the pigment under examination, the other cup with the solution to be examined. The level of the two cups is then altered so that either the intensity of the absorption band is identical in both spectra, or, what gives even more satisfactory results, the absorption band is just on the limit of visibility. The accuracy of the estimation can be increased considerably by interposing filters transmitting only the light of a small region of the spectrum in which the absorption band is situated. By this means Weigert's objection mentioned above is eliminated.

(4) The instrument can be used with advantage for the quantitative estimation of pigments with intense absorption lines or bands if no other pigment is present in the solution. The estimation of concentration by comparison of an absorption band of a solution of unknown concentration with that of a known concentration is frequently preferable to the comparison of the colour intensities of the two solutions, even in the absence of other pigments. Thus, using 4 cm. cups and Wratten filter 58 or 61, or Ilford filter 605, it is possible to estimate coproporphyrin in 25% HCl by using the absorption band at 5502 Å. in 1/2,000,000 solutions, while the absorptiometric estimation is only possible in 1/600,000 solutions. The accuracy of the estimation depends upon the position in the spectral region of the absorption band and its relation to the sensitivity of the human eye to the different parts of the spectrum. By far the most suitable region is that between 5200 and 6000 Å. With the use of suitable filters, and ten readings for each concentration, the average error can be reduced to less than 1%.

(5) The instrument can be used for qualitative comparison of two spectra. This is particularly useful when investigating serial eluates in chromatographic absorption analysis intended to separate pure eluates from those containing two or more pigments. The ease in varying the depths of the layers allows one to see rapidly if an altered spectrum is due only to altered concentration or to the presence of a second pigment in the solution.

Comparison of spectra is also a valuable means of identifying pigments, particularly when the differences in the positions of the absorption bands are slight, e.g. blood pigments, chlorophylls etc. The exact determination of the position of maximum absorption requires comparatively elaborate work with expensive instruments, whereas the comparison of the spectrum of an unknown pigment with that of a known substance in the spectrocomparator reveals immediately its identity or non-identity.

Summary

An instrument is described which combines the qualities of a Duboscq absorptiometer and a spectroscope. It permits simultaneously qualitative and quantitative estimation of certain pigments in the presence of other pigments.

My best thanks are due to the Bausch and Lomb Optical Co., Ltd., and particularly to Mr A. E. Jenkins for the loan of a Duboscq absorptiometer. I wish to express my thanks to the Lister Institute for hospitality and a personal grant. I am much indebted to Dr W. T. J. Morgan for assistance in the preparation of the manuscript, and to Mr H. Ellinger and Mrs A. Moro for preparing the figure.

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31. The Surface Behaviour of Antibacterial Substances

1. Sulphanilamide and Related Substances*

By **Frank R. Bradbury** and **D. O. Jordan**, *From the Chemistry Department, University College, Nottingham*

(*Received 25 February 1942*)

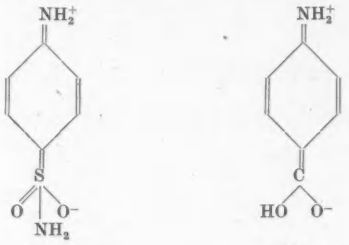
The adsorption of polar molecules at the surface of bacteria will necessarily be accompanied by changes in the charge at the surface. Such an effect may be investigated by electrophoretic methods. The mobility of a small particle when placed in a potential gradient is proportional to the electrokinetic potential, which is dependent on both the surface charge and the thickness of the diffuse layer.

The electric mobility of bacteria has been investigated in connexion with determinations of stability and isoelectric point, and the effect of simple inorganic salts on these quantities has been studied by Abramson [1934] and others. The effect of the age of the bacteria on the electric mobility has been investigated by Moyer [1940], who observed that the mobility of *B. coli* passes through a minimum during the first 7 hr. of growth, thereafter remaining independent of age. In agreement with Moyer we have found 18–36 hr. cultures of *B. coli* to have a mobility independent of age. Mobilities have been determined on suspensions of bacteria in distilled water [cf. Pedlow & Lisse, 1936], but the use of this medium has been criticized by Abramson [1934] and by Moyer [1940] on the grounds that slight changes in ionic concentration produce appreciable changes in mobility, and their experiments have been carried out in buffered salt solutions. Such a method cannot be employed when the effect of small concentrations of polar compounds is being investigated, but by using 'equilibrium water' (conductivity $0{\cdot}6\text{–}1{\cdot}0 \times 10^{-6}$ ohm^{-1} cm.$^{-1}$) we have found it quite possible to obtain reproducible values for the mobility of *B. coli* suspensions (Fig. 6). Any slight variation in the initial reading is of less consequence when observing the variation of mobility with time in the same suspension than in a series of readings on different suspensions.

The interference of antibacterial agents and essential metabolites has been demonstrated by a number of workers. Woods [1940] has shown that sulphanilamide is antagonized by *p*-aminobenzoic acid, and the latter has been shown to be an essential metabolite by Rubbo & Gillespie [1940]. Fildes [1940] has interpreted this antagonism as indicating that the antibacterial substance exerts its characteristic action on the organism by interfering with the utilization of the essential metabolite. This interference may take the form of competition for a common substrate due to the chemical similarity of the molecules concerned, both sulphanilamide and *p*-aminobenzoic acid containing a basic and an acidic group in the *para* position in a benzene ring. Further, from a comparative study of the dipole moments of sulphanilamide, metanilamide, and the esters of *p*- and *m*-aminobenzoic acid, Kumler & Halverstadt [1941] have concluded that the contribution of the forms (I) and (II) to the resonance structure is about the same for sulphanilamide as for *p*-aminobenzoic acid.

* A preliminary report of this work was given at the Biochemical Society discussion meeting on 'Mode of Action of Chemotherapeutic Agents' (see *Nature* [1941], **148**, 757).

It was expected that sulphanilamide and *p*-aminobenzoic acid would behave similarly at the bacterial surface, and that this similarity would reveal itself in the effect of the two compounds on the electric mobility:



(I) (II)

Experimental

A suitable type of single cell for determining electrophoretic mobility has been described by Abramson [1928] and Moyer [1936, 1]. Smith & Lisse [1936] have introduced a new double cell in which the stationary level is at the centre of the cell, and this apparatus has been found to be satisfactory by Powney & Wood [1940] for measurements on paraffin suspensions. We have modified the original design of the Smith & Lisse cell to eliminate rubber joints, by the construction of the all-glass apparatus shown in Fig. 1. The capacity was approximately 5 ml. The visibility of the bacteria was considerably improved by grinding the cell very thin, cementing on parts of a coverslip with canada balsam, and using dark-ground illumination. The apparatus was mounted on the stage of a microscope and the movement of the bacteria observed over a distance of 166 μ, using an eyepiece grating. The potential gradient was calculated by the method of Northrop [1922], and values of 6·4, 12·8 and 19·2 V./cm. were used according to the mobility.

Each measurement of mobility was the mean time of 50 readings on different individual bacteria, the current being reversed after every five readings to prevent polarization. All measurements were made at room temperature, and a filter of saturated alum solution was placed between the cell and the source of illumination to remove heat radiation.

The glass apparatus was cleansed thoroughly between experiments using warm chromic acid followed by thorough washing with water. Traces of sulphanilamide and, in particular, *p*-aminobenzoic acid were not, however, completely removed by this treatment, and prolonged soaking in purified acetone (in which they are remarkably soluble) was found to be satisfactory for cleansing after use of these compounds.

Bacterium coli-commune Esch. (National Collection of Type Cultures, no. 86) was grown in liquid-nutrient broth (pH 7·4) for 18–36 hr. at 37°. The organisms were removed from the medium by centrifuging for 10 min. (3000 r.p.m.), resuspended in conductivity water, centrifuged and again washed, being finally separated after two such washings. In agreement with Moyer [1936, 2] it was found that further washing had no effect on the mobility and was therefore unnecessary.

The washed organisms were suspended in sufficient conductivity water to give an opacity equal to that of a standard $BaSO_4$ suspension [cf. Gardner, 1931]. Counts were made on a Thoma haemocytometer with a chamber 0·1 mm. deep with double Neubauer ruling, giving 400 small squares each $\frac{1}{400}$ mm.^{2} in area. The average count showed the suspension to contain 15×10^7 bacteria per ml. 5 ml. of this suspension and an appropriate volume of a standard solution of the chemical under investigation were diluted to 100 ml. The suspension was drawn into the chemically clean apparatus and mobility measurements taken at intervals of approximately 15 min.

Results and discussion

The all-glass modification of Smith & Lisse's cell has proved a very convenient apparatus for the determination of electrophoretic mobility of bacteria suspensions. Abramson [1940] has indicated some advantages of this type of cell and has suggested that further evidence that it provides quantitative data should be forthcoming. Our experiments have been made with four such cells and two different observers. In every case a value of $3{\cdot}0 \pm 0{\cdot}1\,\mu$/sec./V./cm. has been obtained for *B. coli* suspended in 'equilibrium water', provided that the apparatus was free from traces of chemicals.

All the active antibacterial compounds studied produced a characteristic effect on the mobility of *B. coli*. In contrast to the inactive substances, which produced an initial change in mobility which did not subsequently alter with time of contact, the active substances affected the mobility of the bacteria in a way which varied with the time of contact with the solution of the drug. This change was characterized by the formation of two well defined maxima in the mobility-time curve, as illustrated in Figs. 2 and 3, the bacteria finally attaining a constant or slowly decreasing mobility. Preliminary experiments with proflavine, an antibacterial substance chemically unrelated to the sulphanilamide group, indicate that it too shows this characteristic behaviour. The production of such a mobility-time curve may be an essential requirement for activity, although further investigation will be required to establish this.

In Fig. 2 the variation of mobility with time is shown for solutions of sulphanilamide having various concentrations. The curve for the 0·01 % solution has been determined on four occasions using three different cells. The effect of dilution is to cause a shift of the curve without producing any great change in its characteristic shape, the formation of the maxima, in particular the second, occurring after a greater period of time.

p-Aminobenzoic acid produced an effect on the mobility of *B. coli* closely resembling that of the antibacterial substances (Fig. 4). This may be interpreted as indicating that sulphanilamide and related antibacterials behave in a similar manner to *p*-aminobenzoic acid at the surface of the bacteria. This is in conformity with the view [Fildes, 1940] that sulphanilamide exerts its characteristic action by interfering with the normal utilisation of *p*-aminobenzoic acid. The close similarity of the respective curves suggests that this interference may take the form of competition between sulphanilamide and *p*-aminobenzoic acid for certain reactive centres on the surface of the organism; the drug, by virtue of its similar surface reaction, being able to take the place of the essential metabolite and so exclude it from the bacterial surface. Rubbo & Gillespie [1940] demonstrated that 23,000 molecules of sulphanilamide were required to inactivate 1 mol. of *p*-aminobenzoic acid, which suggests a mass effect of this nature by the drug.

Comparing the curves for benzenesulphonamide and for aniline (Fig. 6) it appears evident that the benzenesulphonamide is without action on the bacterial surface, there being no change of mobility with time of contact with this substance. On the other hand, aniline hydrochloride solution (0·01 %, containing 64 % of the cation $C_6H_5.NH_3^+$) has a marked surface effect, the mobility falling to 75 % of its original value during 4 hr. It may be concluded that the group $-SO_2NH_2$ is without initial effect on the bacterial surface, the first association of the drug molecule and the bacterial surface being due to the positively charged quaternary N. This view receives support from the investigations of Rideal & Schulman [1939] on the relative surface reactivity of various polar groups. Working on surface films of cholesterol they found surface reactivity to be in the order

$$R.NH_3^+ \quad R.SO_4^- \quad R.SO_3^- \quad R.N(CH_3)_3^+ \quad R.NH(CH_3)_2^+,$$

the group $R.NH_3^+$ having the greatest activity. If the first association of the drug molecule with the bacterial surface is due to the presence of a positively charged quaternary N of the type $-NH_3^+$, the necessity for the presence of a free, or potentially

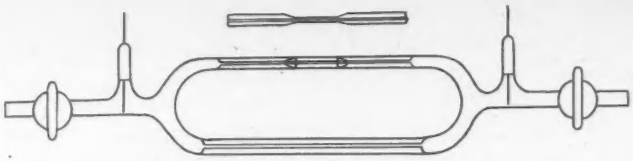


Fig. 1. All-glass electrophoresis cell.

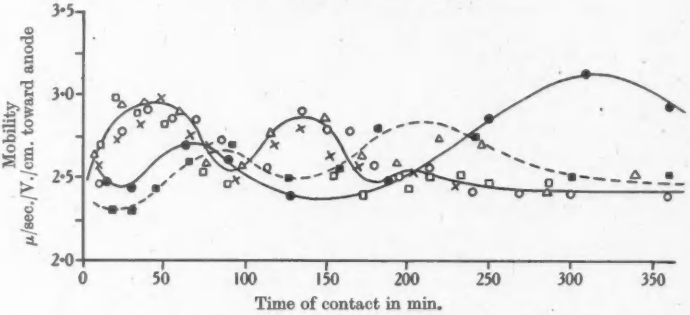


Fig. 2. Variation of mobility of *B. coli* with time of contact with solutions of sulphanilamide. □ ○ △ × 0·01 % solution ■ 0·005 % solution. ● 0·001 % solution.

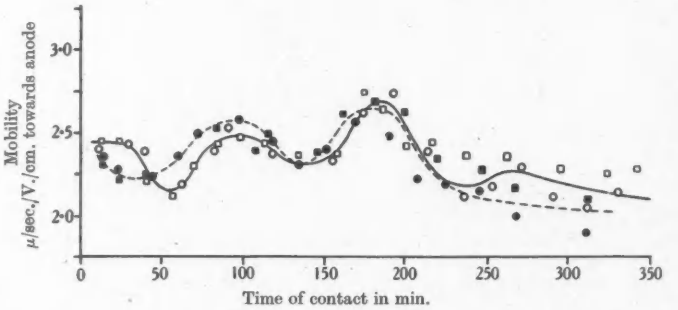


Fig. 3. Variation of mobility of *B. coli* with time of contact with solutions of sulphapyridine and sulphathiazole. □ ○ 0·01 % solution of sulphapyridine. ■ ● 0·01 % solution of sulphathiazole.

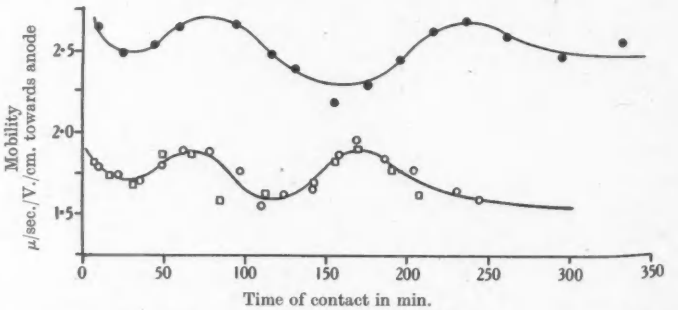


Fig. 4. Variation of mobility of *B. coli* with time of contact with solution of *p*-aminobenzoic acid. □ ○ 0·01 % solution. ● 0·001 % solution.

free, $-NH_2$ group in the molecule of active sulphanilamide derivatives [cf. Northey, 1940, 1] receives explanation.

The curves for N_4-acetylsulphanilamide and for metanilamide are shown in Fig. 5. The former compound shows a very attenuated form of the sulphanilamide curve, the

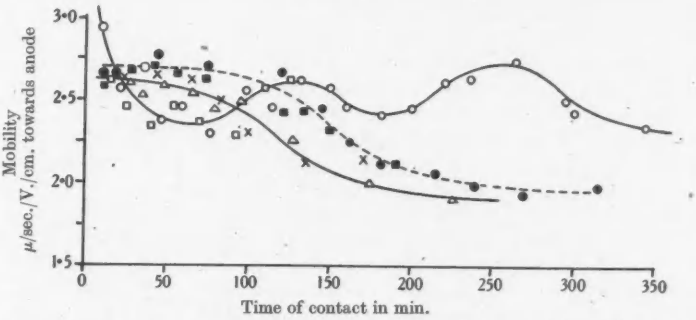


Fig. 5. Variation of mobility of *B. coli* with time of contact with solutions of acetylsulphanilamide, metanilamide and aniline hydrochloride. □ ○ 0·01 % solution of N_4-acetylsulphanilamide. ■ ● 0·01 % solution of metanilamide. △ × 0·01 % solution of aniline hydrochloride.

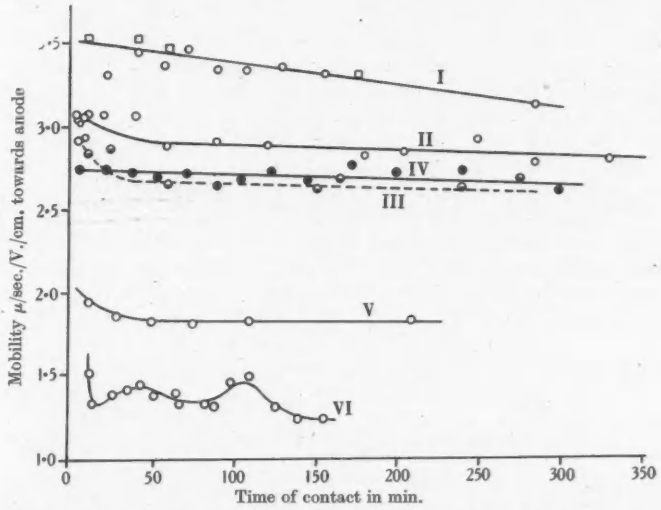


Fig. 6. Variation of mobility of *B. coli* with time of contact with solutions of some inactive substances. I, sodium benzenesulphonate. II, equilibrium water. III, aniline. IV, benzenesulphonamide. V, sulphanilic acid. VI, benzoic acid.

characteristic maxima being more drawn out than those of a 0·001 % sulphanilamide solution (Fig. 2). This corresponds to its known slight activity [Buttle *et al.* 1936]. Metanilamide, a substance without activity [cf. Northey, 1940, 2], behaves very much like aniline hydrochloride. Removal of the sulphonamide group from the position *para*- to the position *meta*- to the $-NH_2$ group has converted a compound exhibiting characteristic maxima into one giving an effect similar to that of aniline. The two substituent

groups evidently behave quite independently when *meta-* to one another, the $—SO_2NH_2$ group having no action on the surface, the $—NH_2$ group producing the slow fall of mobility with time characteristic of anilinium ion. This effect of positional isomerism on the surface properties and activity is paralleled by its effect on resonance within the drug molecule. When the $—SO_2NH_2$ and $—NH_2$ groups are *para-* to one another the polar structure (I) contributes to the resonance of the molecule. When the groups are in the *meta-* position no such polar form is possible. It may be concluded that the polar character of the molecule is intimately connected with its activity. Such polar resonance forms are possible for all the active sulphanilamide derivatives studied as well as for *p*-aminobenzoic acid [cf. Kumler & Halverstadt, 1941].

Whilst this production of polarity by resonance is evidently more important for activity than ampholytic character (metanilamide and sulphanilamide are both ampholytes), other structural properties of such polar molecules must contribute to their antibacterial activity since Kumler & Halverstadt [1941] have shown that in *p*-(*p*-aminophenyl)-benzenesulphonamide the contribution of the polar resonance form is twice as great as in sulphanilamide although the compound is inactive. Also orthanilamide, which could show polar resonance between the $—NH_2$ and $—SO_2NH_2$ groups is without activity [Northey, 1940, 2]. The additional data required to provide a detailed interpretation of the mobility-time curves characteristic of antibacterial substances are being sought by further experiments, which will form the subject of future communications.

Summary

The electrokinetic mobility of suspensions of *B. coli* in dilute aqueous solutions of sulphanilamide, *p*-aminobenzoic acid and chemically related compounds has been determined using an improved all-glass cataphoresis cell.

The effect of sulphanilamide and related antibacterials on the mobility of the organism is quite different from that of inactive substances such as aniline, benzenesulphonamide and metanilamide, but closely resembles that of *p*-aminobenzoic acid. It is concluded that sulphanilamide and related drugs behave like *p*-aminobenzoic acid at the bacterial surface.

The effects of sulphanilamide, benzenesulphonamide and aniline hydrochloride on the mobility have been compared, leading to the conclusion that the association of the drug with the organism is a function of the $—NH_2$ group, and that the polarity produced by resonance of active molecules is one of the factors determining activity.

We are indebted to Prof. J. M. Gulland for his interest in this work and to Messrs Boots Pure Drug Co., Ltd., and Messrs Imperial Chemical Industries, Ltd., for gifts of chemicals.

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Note added 30 May 1942. Albert & Goodacre [1942] have determined the relative acidities and basicities of sulphanilamide and *p*-aminobenzoic acid, and observed that these two substances have approximately the same basic strength but very different acid strengths. This observation provides additional evidence for the conclusion reached above that the association of the drug molecule with the organism is a function of the $-NH_2$ group.

32. The Action of Formaldehyde on the Cystine Disulphide Linkages in Wool

1. The Subdivision of the Combined Cystine into Two Fractions differing in their Reactivity towards Formaldehyde

By **William Robert Middlebrook** and **Henry Phillips**, *From the Wool Industries Research Association, Torridon, Headingley, Leeds, 6*

(*Received 11 February 1942*)

Recent quantitative studies of the reaction of formaldehyde at ordinary temperatures with collagen and keratin (hair) have shown that in buffered solutions up to *p*H 8 containing 1 % HCHO, the terminal amino-groups of lysine combine with HCHO [Bowes & Pleass, 1939, 1, 2, 3; Highberger & Retzsch, 1939]. In solutions of higher *p*H the terminal guanidino-groups of the arginine side-chains also react. When more concentrated solutions of HCHO are used, Highberger & Salcedo [1940] found that the amount of HCHO with which collagen combined approached the limit of 2 mol. HCHO per free basic group.

Bowes & Pleass [1939, 2, 3], finding that deaminated hair combines with HCHO, have suggested that the cystine disulphide linkages of hair may be reduced by HCHO in alkaline solution and that the thiol groups produced may, like those of cysteine [Ratner & Clarke, 1937], condense with HCHO to give combined thiazolidine-4-carboxylic acid. Trotman *et al.* [1928] also noticed a difference in the firmness with which HCHO was held by collagen (skin) and by wool. They found that whereas all the HCHO could be removed rapidly in boiling water from skins, or from wool treated with cold solutions of HCHO, the HCHO fixed by wool at 75° was only removed very slowly. They also found that wool, after treatment with HCHO solutions at 75°, was more resistant to alkalis, and when boiled with dilute H_2SO_4 evolved HCHO until it was disintegrated.

By determinations of disulphide-S, we have found that below *p*H 7·0 at room temperatures HCHO does not react with the disulphide-S of wool. At 70° reaction takes place slowly and is apparently confined to about one-third of the total S. Evidence is put forward that only one S atom of each disulphide-S group that reacts is present in the treated wool as combined thiazolidine-4-carboxylic acid. Unlike ordinary wool [Elsworth & Phillips, 1938, 1; 1941], wool, after reaction with HCHO in this manner, does not give water-stable thiol and *S*-cysteinesulphonate groups with $NaHSO_3$.

Experimental

*The inertness of wool disulphide-S to HCHO at 22° and p*H 6·9

Pieces of loosely woven, thin woollen fabric, prepared from 64–70's wool, were immersed for 48 hr. in 50 times their weight of 0·017 *M* Na_2HPO_4 at 22° containing 1 % HCHO; the *p*H was 6·9. After treatment, the wool was rinsed in water, dried at ordinary temperature and then conditioned.* The disulphide-S contents of the treated and untreated wools, determined by Shinohara's method [1935, 1], were respectively 2·81 and 2·80 %.† These results show clearly that at *p*H 6·9 and 22° HCHO does not react with the disulphide-S of wool, although Bowes & Pleass [1939, 1] found that hair exposed to an unbuffered 0·8 % solution of HCHO at *p*H 6·8 for 48 hr. fixed about 0·8 % of its weight of HCHO.

* All the wools described in this paper as conditioned had been exposed to a controlled atmosphere of 21° and 70 % R.H. until they attained constant weight.

† Unless otherwise stated, all analytical results are calculated on the weight of the anhydrous wool.

A further experiment showed that at higher *p*H some reaction with the disulphide-S occurs. Degreased, woven fabric (15 g.), which had been brought to equilibrium with water at *p*H 9, was immersed for 48 hr. in 1600 ml. of buffer of *p*H 9 containing 0·8 % HCHO. The buffer was 0·4 *M* in potassium dihydrogen phosphate, phenylacetic acid and boric acid and was brought to *p*H 9 by 0·2 *N* NaOH. The treated wool was rinsed thoroughly with water and then conditioned. By Bowes & Pleass's method [1939, 1] it was found to contain 1·17 % HCHO. The disulphide-S content was 2·75 % as compared with 2·94 % for the untreated wool. At *p*H 9·0 therefore the disulphide-S of wool reacts slowly with HCHO.

Separate experiments showed that HCHO did not interfere with Shinohara's method [1935, 1] for the determination of disulphide-S. Samples of wool were hydrolysed with 5*N* HCl and also with 5*N* HCl containing 0·8 % HCHO calculated on the weight of wool taken for hydrolysis. The determination of the disulphide-S in both hydrolysates gave the disulphide-S content of the wool as 2·80 %.

The action of $NaHSO_3$ on formaldehyde-treated wools

Wool, as loosely woven flannel, was treated with 50 times its weight of 1 % HCHO for 1 hr. at 17°. Another sample of the same wool was treated at 70°. The *p*H of the solutions during the treatment varied between 4·6 and 7·2. After treatment, the wools were rinsed in water and dried at room temperature. They were then immersed for 17 hr. in 40 times their weight of cold aqueous $NaHSO_3$ (*p*H 5·6) containing 3·0 % SO_2. The bisulphited wools were rinsed thoroughly in water, dried at room temperature and then conditioned. Their disulphide and thiol-S contents were determined by Shinohara's methods [1935, 1, 2, 3) and the total, 'free' and combined, SO_2 content by Elsworth & Phillips's methods [1938, 2]. Experiment showed that the addition of HCHO to a bisulphited wool before analysis by these methods did not affect the determination of the different classes of SO_2 which it contained.

Table 1. *The action of $NaHSO_3$ on wool after treatment with 1 % HCHO* (p*H 4·6–7·2*) *for 1 hr. at 18° and at 70°*

Wool treated with HCHO:	Total SO_2 %	'Free' SO_2 %	Combined SO_2 %	Thiol-S %	Disulphide-S %
At 18°	1·18	0·50	0·68	0·42	1·98
At 70°	0·42	0·47	0·00	0·06	1·89

The disulphide-S content of the untreated wool was 2·80 %. From Table 1 it will be seen that this decreased to 1·89 %, when the wool was treated with HCHO at 70° and then the wool did not react with the $NaHSO_3$. On the other hand, after treatment with HCHO at 18°, the wool reacted with $NaHSO_3$ and its disulphide-S was lowered to 1·98 %. Treatment of the wool with either HCHO at 70° or with $NaHSO_3$ under the conditions of these experiments thus decreases the disulphide-S of the wool by approximately the same amount.

The influence of the length of time of treatment of wool with (a) unbuffered, (b) buffered solutions of HCHO at 70° on the subsequent action of $NaHSO_3$ on the wool

(*a*) *Unbuffered solutions.* The wool was immersed for the periods given in Table 2 in 50 times its weight of 1 % HCHO at 70°; the *p*H varied from 4·6 to 7·2 during the treatment. It was then rinsed in water, dried at room temperature and then conditioned. After the disulphide-S contents of the different samples had been determined, they were immersed for 17 hr. in 40 times their weight of aqueous $NaHSO_3$ (*p*H 5·6) at 70° containing 3·0 % SO_2. The bisulphited wools were rinsed thoroughly in water, and before being analysed by the methods previously mentioned, were dried at room temperature and conditioned. The results are given in Table 2.

(*b*) *Buffered solutions.* The above experiment was repeated using a 1 % solution of HCHO 0·02 *M* in NaH_2PO_4; the *p*H was 6·7–6·9. Samples of the formaldehyde-treated wools were immersed in $NaHSO_3$ under two sets of conditions: at the boil for 30 min. in 40 times their weight of aqueous $NaHSO_3$ at *p*H 5·8 containing 1 % SO_2; at room temperature for 24 hr. in 40 times their weight of aqueous $NaHSO_3$ at *p*H 5·5 containing 3·08 % SO_2. Analyses of the bisulphited wools are given in Table 2.

Table 2. *The decrease in the disulphide-S of wool on treatment with* (*a*) *unbuffered, and* (*b*) *buffered solutions of HCHO at 70° and its effect on the wool with* $NaHSO_3$

(*a*) Treatment with unbuffered solutions

Time of treatment with HCHO hr.	Disulphide-S %	After treatment with $NaHSO_3$ at 70°		
		Disulphide-S %	Thiol-S in hydrolysate %	Combined SO_2 %
0	2·80	1·94*	0·83*	—
0·17	2·81	2·11*	0·35*	0·32*
1·0	2·67	2·26	0·09	—
5·0	2·51	2·19	0·19	0·19
48·0	2·17	2·24	—	—
72·0	2·00	—	—	—

(*b*) Treatment with buffered solutions

Time of treatment with HCHO hr.	Disulphide-S %	After treatment with $NaHSO_3$			
		½ hr. at 100° (1 % SO_2 : *p*H 5·8)		24 hr. at room *t*° (3·08 % SO_2 : *p*H 5·5)	
		Disulphide-S %	Thiol-S in hydrolysate %	Disulphide-S %	Thiol-S in hydrolysate %
0	2·80	1·94	0·66	1·94	0·83
3	2·65	2·02	0·49	2·06	0·18
18	2·49	2·17	0·34	1·92	0·16
48	2·12	2·10	0·28	2·02	0·03
96	—	—	—	1·91	0·02
120	1·98	2·03	0·21	—	—

* After treatment with $NaHSO_3$ at room temperature.

The influence of HCHO on the determination of thiol and disulphide-S by Shinohara's methods

As mentioned previously, it was shown that wool could be hydrolysed in the presence of HCHO without affecting the accuracy of the determination of disulphide-S by Shinohara's method [1935, 1]. Further experiments were, however, necessary to test the accuracy of Shinohara's methods when applied to the determination of thiol-S [1935, 2, 3] and disulphide-S [1935, 1] in wools which had reacted with both HCHO and $NaHSO_3$, since the hydrolysates of such wools contain HCHO, cysteine and possibly thiazolidine-4-carboxylic acid. That some irregularities arise is shown by the analyses given in Tables 1 and 2. For example, only 0·42 % of thiol-S was found in the hydrolysate of the wool which had been treated with HCHO at 18° and then with $NaHSO_3$. Since each disulphide group which reacts with $NaHSO_3$ gives rise to two thiol groups when the wool is hydrolysed with HCl, the weight of thiol-S in the hydrolysate should equal the weight of combined SO_2. The combined SO_2 in this wool (Table 1) was 0·68 %, and hence it can be concluded that the thiol-S determination is incorrect, since 0·68 % thiol-S should have been found instead of only 0·42 %. Similarly, in Table 2, it will be seen that the sum of the thiol- and disulphide-S in the hydrolysates of the bisulphited wools is less than the amount of disulphide-S in the wools after treatment with HCHO.

Ratner & Clarke [1937] have shown that whereas cysteine in dilute HCl combines very slowly with HCHO, in solutions at *p*H 5 the reaction is very rapid. In Shinohara's method for the determination of thiol-S the hydrolysate is brought to *p*H 5, phosphotungstic acid is added immediately and the colour is allowed to develop for 20 min. Under these conditions, the length of time the cysteine and HCHO are free to react is short. If, on the other hand, some time is allowed to elapse before the phosphotungstic acid is added, then the cysteine and HCHO will combine and a less intense colour will be developed. For example, a bisulphited wool hydrolysate containing 1·0% thiol-S (calculated on the weight of wool hydrolysed) was boiled with 1·0% HCHO (also calculated on the weight of wool hydrolysed) for 1 hr. When phosphotungstic acid was added immediately after the hydrolysate had been buffered to *p*H 5, the coloration obtained corresponded to 0·95% thiol-S, whereas when the acid was added after the buffered hydrolysate had stood for 1 hr., the coloration developed corresponded to only 0·56% thiol-S.

In order to measure the effect of the HCHO concentration on the thiol-S determinations, samples of a bisulphited, but incompletely rinsed, wool were hydrolysed in the presence of increasing quantities of HCHO, and the thiol-S contents of the hydrolysates were determined after they had been buffered at *p*H 5 for 1 hr.

A further sample of this wool was hydrolysed and increasing quantities of HCHO were added to portions of the hydrolysate buffered at *p*H 5 and determinations of thiol-S were made after 1 hr. The thiol-S contents of these two sets of hydrolysates are given in Table 3. The analyses (Table 3) show that the amounts of thiol-S returned steadily decreased

Table 3. *The effect of HCHO on the thiol-S contents of the hydrolysates of bisulphited and rinsed wools*

HCHO on weight of wool hydrolysed %	(*a*) HCHO added before hydrolysis	(*b*) HCHO added after hydrolysis
0·0	1·37	0·89
0·25	1·01	0·61
0·50	—	0·48
1·0	0·42	0·34
3·0	0·00	—

as the concentration of HCHO increased, but the rate of decrease was the same whether the wool was hydrolysed in the presence of HCHO or whether the HCHO was added after hydrolysis. It is also evident that relatively large concentrations of HCHO must be present before the thiol-S in the wool hydrolysates of the nature we have examined would fail to give any coloration with phosphotungstic acid. Nevertheless, because of the presence of HCHO, the analyses of thiol-S in the formaldehyde-treated and then bisulphited wools (Table 2) are not of a high degree of accuracy. This lack of accuracy, however, does not invalidate the conclusions drawn, since these are based on the analyses of completely formaldehyde-treated wools that do not react with $NaHSO_3$ and did not therefore contain thiol-S.

A further possible loss of accuracy may be caused by the presence of thiazolidine-4-carboxylic acid groupings in formaldehyde-treated wools, since the thiazolidine-4-carboxylic acid liberated by hydrolysis may dissociate into cysteine and HCHO. Experiments were therefore made to test the stability of thiazolidine-4-carboxylic acid under the conditions used for the determination of thiol-S. The acid (150·6 mg.), M.P. 196–197°, prepared by Ratner & Clarke's method [1937], was heated under reflux for 4 hr. with 20 ml. 5*N* HCl in a flask fitted with a long air condenser. A thiol-S determination on the resulting solution showed that 5·5 mg. or 3·6% of the acid had dissociated into cysteine and HCHO. When a solution of the acid (37 mg.) in 20 ml. of 5*N* HCl containing 1·0 g. of thiol-free gelatin was heated under reflux for 4 hr., 11·4% of the acid dissociated. The

possible effect of the presence of combined thiazolidine-4-carboxylic acid in formaldehyde-treated wools on the thiol-S determinations is not likely therefore to have been large. For example, the disulphide-S content of a virgin Cape wool, purified as described by Elsworth & Phillips [1941], was reduced from 3·25 to 2·23 % by treatment with HCHO at 70°. Assuming that all the disulphide-S that disappeared became linked in thiazolidine-4-carboxylic acid groups, then the hydrolysate of 1·0 g. of this wool would contain 37 mg. of the acid. If, during hydrolysis, this acid behaved in the same manner as the acid hydrolysed in the presence of 1·0 g. of gelatin, then it would give rise to only 0·12 % thiol-S.

Since thiazolidine-4-carboxylic acid is decomposed by sulphite [Ratner & Clarke, 1937], it appeared more probable that this acid would interfere with the determinations of disulphide-S. Experiments were therefore made to obtain some measure of this interference. The results were of interest since they provided indirect evidence of the presence of thiazolidine-4-carboxylic acid in formaldehyde-treated wools. In these experiments advantage was taken of the slowness of the reaction between the acid and sulphite at *p*H 5. A virgin Cape wool which had been treated with HCHO at 70° was hydrolysed and the hydrolysate was buffered at *p*H 5 and then treated with sulphite as in Shinohara's method [1935, 1] for the determination of disulphide-S. The amount of cysteine the solution contained was then determined at intervals. Two 'synthetic' hydrolysates were also prepared and their disulphide-S contents determined at intervals in a similar manner. These hydrolysates were obtained by hydrolysing a quantity of untreated wool containing the same amount of disulphide-S as the formaldehyde-treated wool from which the first hydrolysate was prepared. Before hydrolysis was started, however, thiazolidine-4-carboxylic acid equivalent to half the difference between the total-S and disulphide-S of the formaldehyde-treated wool was added to one lot of the untreated wool, and to the other, thiazolidine-4-carboxylic acid equivalent to the difference between the total S and disulphide-S of the formaldehyde-treated wool. Further, since such wools would contain about 0·7 % HCHO linked to basic groupings, HCHO equivalent to 0·7 % of the weight of untreated wool hydrolysed was added to each solution before hydrolysis was started.

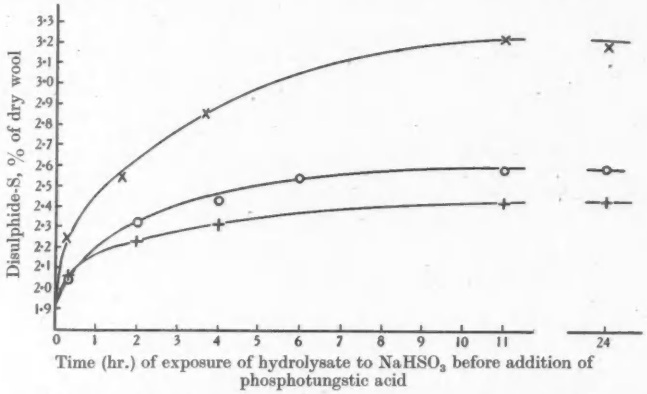


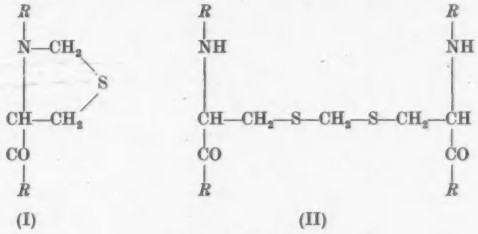
Fig. 1. ×—× Wool + HCHO + 33·9 mg. thiazolidine-4-carboxylic acid. o—o Formaldehyde-treated wool. +—+ Wool + HCHO + 17·0 mg. thiazolidine-4-carboxylic acid.

The results of the disulphide-S determinations on these three hydrolysates are plotted in Fig. 1 against the length of time the hydrolysate at *p*H 5 reacted with bisulphite before phosphotungstic acid was added and the determinations were made. If the increase in disulphide-S with time, which is shown by all the hydrolysates, can be taken to indicate dissociation of thiazolidine-4-carboxylic acid in the presence of sulphite, then it is evident

that the behaviour of the hydrolysate of the formaldehyde-treated wool is more closely imitated by the 'synthetic' hydrolysate containing the smaller quantity of the acid. In other words, the results suggest that only half the cystine-S which is lost when wool is treated with HCHO becomes part of a thiazolidine-4-carboxylic acid grouping. The analytical results support this suggestion closely. Thus the curve (Fig. 1) giving the disulphide-S content of the hydrolysate of the formaldehyde-treated wool, when extrapolated gives the disulphide-S at zero time as 1·92 %. The untreated wool contained 3·25 % disulphide-S. Hence 1·33 % disulphide-S has disappeared during the treatment of the wool with HCHO. Exactly half of this disulphide-S (0·66 %) is regained as thiol-S by exposing the hydrolysate to $NaHSO_3$ for 24 hr.

The curve relating to the hydrolysate of the formaldehyde-treated wool in Fig. 1 enables a small correction to be made to the determinations of disulphide-S recorded in Table 2. These determinations were made 20 min. after the addition of bisulphite to the buffered hydrolysates. From Fig. 1 it will be seen that at zero time the value for the percentage of disulphide-S is 0·10 less than the value at 20 min. The values for the disulphide-S contents of the completely formaldehyde-treated wools given in Table 2 are therefore greater by 0·10 than the true values.

Having obtained evidence of the presence of thiazolidine-4-carboxylic acid in formaldehyde-treated wool, it is interesting to note that the acid, as combined in wool, is relatively stable to $NaHSO_3$. This is shown by the failure to find thiol-S in formaldehyde-treated wools after they had been subjected to aqueous $NaHSO_3$ under various conditions. Even when such wools were immersed in boiling $NaHSO_3$ for 30 min. (Table 2) only small quantities of thiol-S were produced (0·21 %), being only slightly greater than the amounts produced (0·15 %) when the cold hydrolysate had been in contact with $NaHSO_3$ for 30 min. (Fig. 1). This is in agreement with the conclusion of Ratner & Clarke [1937] that acetylthiazolidine-4-carboxylic acid is much more stable to $NaHSO_3$ than is the unsubstituted acid. It thus provides some evidence that HCHO combines with wool as in (I).



(I) (II)

R = polypeptide chain.

The supercontraction of formaldehyde-treated hair

Instead of condensing as in (I), the disulphide-S and HCHO may condense and produce djenkolic acid [Van Veen & Hyman, 1935] combined as in (II). The properties of this acid are similar to those of thiazolidine-4-carboxylic acid, although some doubt exists as to its stability towards HCl; Van Veen & Hyman [1935] reported that no cleavage occurred when it was boiled with varying concentrations of HCl, whereas Lillevik & Sandstrom [1941] state that it is decomposed into cysteine and HCHO to the extent of 85 % when boiled for 24 hr. with 20 % HCl.

The formation of combined djenkolic acid in this manner would produce stable cross-linkages between the polypeptide chains, and would explain the observed resistance of formaldehyde-treated wool to enzymic attack. For example, we have found that, although wool after treatment with HCHO at room temperature is rapidly disintegrated at 65°

by a solution of papain in $NaHSO_3$ [Middlebrook & Phillips, 1941], wool after treatment with HCHO at 70° is resistant to this enzyme solution.

The results of supercontraction experiments were, however, not in agreement with the presence of stable cross-linkages in formaldehyde-treated hair. Speakman [1936] found that when hair is boiled for 30 min. with 5% $NaHSO_3$, it supercontracts 30% in length when dried. Chemical treatments of hair, such as boiling with alkaline solutions [Speakman & Stoves, 1937], reduce the extent of the supercontraction, when the hair is subsequently boiled with $NaHSO_3$. It is considered [Speakman & Stoves, 1937] that this is due to the cross-linking of the polypeptide chains by stable covalent linkages, derived from the interaction of the disulphide-S with adjacent groupings. On this hypothesis, djenkolic acid cross-linkages of the type indicated in (II) would inhibit the supercontraction of keratin fibres. The supercontraction of degreased human hairs after treatment with HCHO at room temperature and at 70°, under the conditions given in Table 4, were therefore studied. The treated hairs were boiled in 5% $NaHSO_3$ for 30 min., and the extent to which they contracted was measured before and after they had been air-dried.

Table 4. *The supercontraction of formaldehyde-treated hair when boiled in 5% $NaHSO_3$ for 30 min.*

	Supercontraction %			
	Untreated	1% HCHO (*p*H 5·6) at room temp. for 48 hr.	1% HCHO (*p*H 9·0) at room temp. for 48 hr.	1% HCHO (*p*H 5·6) at 70° for 48 hr.
Wet	9·8	4·3	8·6	12·8
Dry	21·0	22·4	27·6	23·0

It is evident from these results that pre-treatment of hair either at room temperature or at 70° does not alter its power to supercontract when boiled with 5% $NaHSO_3$. On the basis of the hypothesis that cross-linkage formation would prevent supercontraction, it appears that djenkolic acid is not produced (as in (II)) when hair reacts with HCHO. It might be suggested that djenkolic acid cross-linkages would be destroyed when the hair is boiled with $NaHSO_3$, but the analyses of formaldehyde-treated wool that has been boiled with $NaHSO_3$ give little support to this suggestion.

The influence of p*H on the rate of reaction of wool with buffered solutions and with buffered solutions containing HCHO*

It is probable that HCHO does not react directly with the disulphide-S of wool, but as in the case of $NaHSO_3$ [Elsworth & Phillips, 1938, 1] reacts with the products of its hydrolysis:

$$—CH_2S.S.CH_2— \rightleftharpoons —CH_2SH + HOS.CH_2—$$

At room temperature, the rate of production of combined thiazolidine-4-carboxylic acid is slow, but at 70° and *p*H 5·6 it proceeds at a measurable rate. Even in the absence of HCHO, however, relatively large decreases in the disulphide-S of wool can be produced which become greater as the *p*H of the solution increases. Elsworth & Phillips [1941] showed, for example, that after wool had been boiled with a buffer at *p*H 8·5 for 30 min., it contained 1·06% of non-disulphide-S* produced without loss of S. The loss of disulphide-S which occurs when wool is treated with solutions of HCHO at 70° is thus the result of two changes: the conversion of disulphide-S into non-disulphide-S, and the interaction of some of the thiol groups produced from the disulphide-S with HCHO. In order to compare the extents of these two changes, samples of Cape wool were immersed for increasing

* The term non-disulphide-S is used to describe S not returned either as disulphide-S or thiol-S by Shinohara's methods [1935, 1, 2, 3] or as methionine-S by Baernstein's method [1932].

periods in 50 times their weight of buffer solutions at *p*H 5·6 (0·1 *M* sodium acetate) and 8·5 (0·15 *M* borax with boric acid), and also in similar solutions containing 1·0 % HCHO. These solutions were kept at 70°, and the treated samples of wool, after rinsing, were analysed for disulphide-S. The results are plotted against the length of time of treatment in Fig. 2. It will be seen that in buffer solutions at *p*H 5·6, the disulphide-S content of the

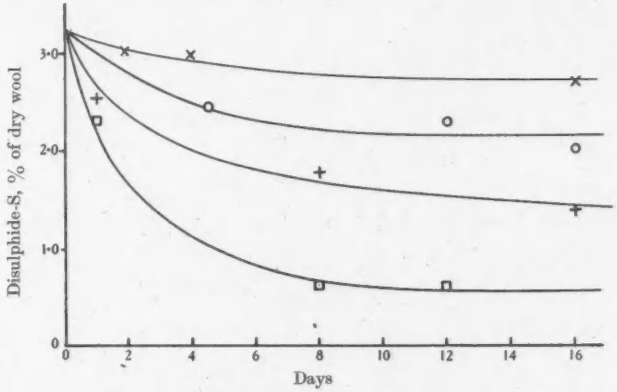


Fig. 2. ×—× Buffer *p*H 5·6. o—o Buffer *p*H 5·6 containing 1 % HCHO. +—+ Buffer *p*H 8·5. □—□ Buffer *p*H 8·5 containing 1 % HCHO.

wool changed slowly and reached a constant value of about 2·7 %. When the buffer contained HCHO, the disulphide-S fell more rapidly and tended to reach a constant value of 2·0 %. In buffer at *p*H 8·5, the disulphide-S of the wool fell much more rapidly and the rate of fall was maintained, until the value 1·4 % was reached. The presence of HCHO increased the rate of disappearance of disulphide-S which had fallen to 0·5 % in 16 days.

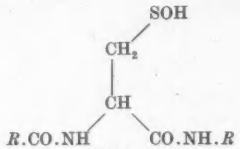
Discussion

Elsworth & Phillips [1928, 1, 2; 1941] showed that approximately one-third of the disulphide-S of wool reacts with $NaHSO_3$ to give thiol and *S*-cysteinesulphonate groups which persist when the wool is rinsed with water. The present investigation has shown that these groups are not produced by the action of $NaHSO_3$ on wool which has been treated with HCHO at 70°. It thus appears that in solutions at *p*H 5·6 only about one-third of the disulphide-S of wool reacts with both $NaHSO_3$ and HCHO. Above *p*H 5·6, a larger proportion of the disulphide-S of wool is changed. Although this may be due partly to increased reaction with HCHO, it is mainly due to reaction of the disulphide-S with alkali. This leads to the production of non-disulphide-S with the simultaneous disappearance of an equivalent amount of disulphide-S.

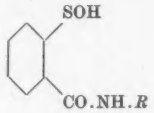
Should hydrolysis of the disulphide-S linkage precede condensation with HCHO, the formation of either combined djenkolic or thiazolidine-4-carboxylic acid involves assumptions as to the fate of the sulphenic acid group (III). If the HCHO condenses with the thiol group, the $—S.CH_2.OH$ group produced can only yield combined djenkolic acid, when the sulphenic acid group has been reduced to a thiol group. On the other hand, if the thiol group, produced by hydrolysis of the disulphide-S group, condenses with HCHO to give combined thiazolidine-4-carboxylic acid, then either the sulphenic acid group remains free or reacts with one or more adjacent groups.

There is no evidence that the sulphenic acid group is reduced under the conditions of our experiments. Further, it is unlikely that the sulphenic acid group remains free and un-

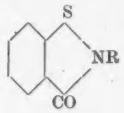
changed, since it would then persist in the acid hydrolysates of formaldehyde-treated wool. Here it would be detected by its reducing action on the phosphotungstic acid reagent [Clarke, 1932]. No such reducing action was observed. On the assumption therefore that one S atom only of the disulphide-S group condenses with HCHO, the fate of the S atom of the sulphenic acid group remains obscure, the only likely suggestion that might be made at present being that this group condenses with the adjacent imino-group of the polypeptide chain in the same manner as the sulphenic acids derived from substituted amides (IV) condense to give ketobenz*iso*thiazoles (V) [McClelland & Warren, 1930].



(III)



(IV)



(V)

R = polypeptide chain.

Summary

1. At 70°, but not at room temperature, wool reacts with HCHO in solutions at *p*H 5·6, and combined thiazolidine-4-carboxylic acid equivalent to half the disulphide-S that disappears is probably produced. Only about one-third of the total disulphide-S of wool reacts in this manner.

2. Wool that has reacted with HCHO at 70° does not react with $NaHSO_3$ to give thiol and *S*-cysteinesulphonate groups that are stable to water.

3. It is probable that the fraction of the disulphide-S of wool which reacts with $NaHSO_3$ to give water-stable thiol and *S*-cysteinesulphonate groups is also the fraction of the disulphide-S that reacts with HCHO at *p*H 5·6 and 70°.

Thanks are due to the Council of the Wool Industries Research Association for permission to publish this paper.

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33. The Effect of Inorganic Salts on the Ketone Decomposition of Oxaloacetic Acid

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Oxaloacetic acid is known to decompose in aqueous solution into pyruvic acid and CO_2. At *p*H 4 and 20° the rate of the 'ketone decomposition' varies between 6 and 18 % per hr. The fact that this 'spontaneous' decomposition shows considerable variations suggests that trace impurities might affect the stability of oxaloacetic acid. Krampitz & Werkman [1941] recently reported that Mg ions accelerate the decomposition; this makes it probable that the impurities in question are inorganic salts. The effects of various salts on the rate of decomposition were therefore studied.

Oxaloacetic acid was prepared from tartaric acid according to the methods of Wohl & Oesterlin [1901] and Wohl & Claussner [1907], and recrystallized according to Fenton & Jones [1900]. The rate of decomposition was measured by the manometric determination of the CO_2 evolution.

Table 1 shows that many inorganic salts increase the rate of decomposition. Small quantities, amounting to 0·3 % of the oxaloacetic acid added, are in many cases sufficient to produce a pronounced effect. The effect is thus a catalytic one. The data in Table 1 suggest the following details:

Table 1. *Effect of inorganic salts on the rate of ketone decomposition of oxaloacetic acid*

0·02 *M* oxaloacetic acid in 3 ml. 0·3 *M* acetate buffer *p*H 5·0; 40°; the salts were added from a side-arm when the solution had been shaken at 40° for 15 min. The data on CO_2 evolution refer to the second 5 min. period after the additions.

Substrate added	Final molarity	μl. CO_2 evolved in 5 min.	Substrate added	Final molarity	μl. CO_2 evolved in 5 min.
None	—	48	$MnSO_4$	$6·6 \times 10^{-3}$	372
NaCl	$6·6 \times 10^{-3}$	49	$CdSO_4$	$6·6 \times 10^{-3}$	223
KCl	$6·6 \times 10^{-3}$	48	$FeSO_4$	$6·6 \times 10^{-3}$	622
KH_2PO_4	$6·6 \times 10^{-3}$	47	,,	$0·66 \times 10^{-3}$	389
Na_2SO_4	$6·6 \times 10^{-3}$	44	,,	$0·066 \times 10^{-3}$	166
$CaCl_2$	$6·6 \times 10^{-3}$	72·5	Pb-acetate	$6·6 \times 10^{-3}$	730
$MgCl_2$	$6·6 \times 10^{-3}$	132	,,	$0·66 \times 10^{-3}$	159
$BaCl_2$	$6·6 \times 10^{-3}$	49	$Fe_2(SO_4)_3$	$6·6 \times 10^{-3}$	600
$ZnSO_4$	$6·6 \times 10^{-3}$	460	$Al_2(SO_4)_3$	$6·6 \times 10^{-3}$	1300
$AgNO_3$	$6·6 \times 10^{-3}$	48	,,	$0·66 \times 10^{-3}$	364
$HgCl_2$	$6·6 \times 10^{-3}$	48	,,	$0·066 \times 10^{-3}$	178
$CuSO_4$	$6·6 \times 10^{-3}$	732	$K_3Fe(CN)_6$	$6·6 \times 10^{-3}$	45
,,	$0·66 \times 10^{-3}$	660	$K_4Fe(CN)_6$	$6·6 \times 10^{-3}$	54
,,	$0·066 \times 10^{-3}$	289			

(1) The catalytically active principle is associated with the cations. Anions are inactive.

(2) Univalent cations (Na, K, Ag) are inactive.

(3) All the multivalent cations tested were found active. The inactivity of $HgCl_2$ is no exception as the solution of this salt contains no significant amounts of Hg ions.

(4) No clear rule emerges as yet regarding the factor determining the degree of activity of the various cations. Among the tested substances Al, Cu, Fe II and Fe III are the most powerful catalysts at *p*H 5·0.

Effect of H^+ concentration. In strongly acid solution (*p*H 1 and below) oxaloacetic acid shows no measurable rate of decomposition at 20°. At 40° the decomposition is very

slight. Addition of $Al_2(SO_4)_3$ (10^{-2} *M*) has no appreciable effect. In strongly alkaline solution (*p*H 13) oxaloacetic acid is equally stable. The optimum rate of decomposition is not sharply defined; it is near *p*H 4 (Table 2).

Table 2. *Effect of the H^+ concentration on the rate of decomposition of oxaloacetic acid in the presence of $Al_2(SO_4)_3$*

3 ml. medium; 20°; 0·02 *M* oxaloacetic acid; $0·67 \times 10^{-4}$ *M* $Al_2(SO_4)$; phthalate buffer 0·2 *M*; acetate buffer 0·6 *M*.

*p*H	Buffer	µl. CO_2 evolved in 20 min.	*p*H	Buffer	µl. CO_2 evolved in 20 min.
0·0	*N* HCl	7·5	4·4	Acetate	63
1·2	Phthalate	29·5	4·7	,,	53
1·7	,,	48	5·1	,,	47·5
2·0	,,	60	5·7	,,	22
3·8	,,	58			

Reactivity of other ketonic acids. The stability of acetoacetic acid, mesoxalic acid, α-ketoglutaric acid and pyruvic acid is not influenced by the addition of Al salts, whilst acetonedicarboxylic acid is rapidly split, as shown in Tables 3 and 4. One molecule of CO_2 is rapidly liberated from acetonedicarboxylic acid; this is followed by the slow evolution of a second molecule. The rate of formation of the second molecule is the same as that of the 'spontaneous' decomposition of acetoacetic acid. Thus Al ions catalyse the conversion of acetonedicarboxylic acid into acetoacetic acid and CO_2, but have no effect on the ketone decomposition of acetoacetic acid.

Table 3. *Decomposition of acetonedicarboxylic acid by $Al_2(SO_4)_3$ and aniline*

Medium: 3 ml. 0·1 *M* acetate buffer *p*H 4·4; 20°; 0·0224 *M* acetonedicarboxylic acid. The catalysts were added after thermal equilibration.

Time (min.)	Catalysts added...	0·5 ml. 0·1 *M* $Al_2(SO_4)_3$	1·0 ml. aniline citrate
		µl. CO_2 evolved	
5	8·5	122	496
10	19	260	948
20	33	448	1300
35	54	608	1472
55	81	707	1490
95	121	745	1502
135	167	790	1508
160	180	802	1512

Table 4. *Relative rates of decomposition of oxaloacetic and acetonedicarboxylic acids*

20°. The data refer to 3 ml. solution. Medium: 0·1 *M* acetate buffer, *p*H 4·4.

Substances added...	0·02 *M* oxaloacetic acid	0·02 *M* oxaloacetic acid 3×10^{-3} *M* $Al_2(SO_4)_3$	0·02 *M* acetone-dicarboxylic acid	0·02 *M* acetone-dicarboxylic acid 3×10^{-3} *M* $Al_2(SO_4)_3$
Time (min.)		µl. CO_2 evolved		
10	19	213	22	41
20	39	422	40	76
30	59	626	56	119

It follows that the catalytic effect of the cations is limited to β-ketonic-dicarboxylic acids. α-Ketonic-dicarboxylic acids, or β-ketonic-monocarboxylic acids do not react. Amines, on the other hand, split all β-ketonic acids [Ljunggren, 1925] and aniline therefore liberates 2 mol. of CO_2 from acetonedicarboxylic acid (Table 3).

Qualitative test for oxaloacetic acid. In testing the effect of ions on oxaloacetic acid it was noted that $HgNO_3$ forms a precipitate with oxaloacetic acid which is still distinctly visible when 0·01 mg. oxaloacetic acid, dissolved in 3 ml. water, is mixed with 1 ml.

0·1 *N* $HgNO_3$. The precipitate is white in dilute solution and dark grey in more concentrated solutions of oxaloacetic acid. It is soluble in HNO_3 (unlike mercurous fumarate). It disappears on prolonged standing, or on heating, owing to the ketone decomposition of oxaloacetic acid. Acetonedicarboxylic acid forms a similar precipitate. Pyruvic and α-ketoglutaric acids do not react. The reaction can be used to detect small quantities of oxaloacetic acid in the presence of pyruvic acid, provided that other substances forming insoluble mercurous compounds (e.g. Cl ions) are absent.

Purification of oxaloacetic acid. Efforts to prepare stable solutions of oxaloacetic acid by repeated recrystallization or by treatment of the solutions with metal-binding reagents (dithizone; 8-hydroxyquinoline) were unsuccessful. Under the test conditions (0·2 *M* acetate buffer *p*H 4·0; 20°; 0·02 *M* oxaloacetic acid) the rate of decomposition did not fall below 6% per hour. It would therefore appear that the 'spontaneous' ketone decomposition of oxaloacetic acid is partly due to the inherent instability of the grouping $R.CO.CH_2.COOH$.

Discussion

Two different types of catalysts—amines and multivalent cations—are now known to decompose ketonic acids; the former react with all β-ketonic acids and in certain cases with α-ketonic acids [Langenbeck, 1933]; the latter only with β-ketonic-dicarboxylic acids.

The catalysts concerned with these reactions in biological systems seem to involve cocarboxylase (an amine) and/or Mg (a divalent cation) [see Krampitz & Werkman, 1941]. Details of the biological mechanisms are still obscure; it may well be that forces similar to those acting in the 'model' systems are effective in the biological systems.

Summary

A number of multivalent cations (Al, Cu, Fe II, Fe III, etc.) are shown to catalyse the ketone decompositions of oxaloacetic and of acetonedicarboxylic acids. The cations do not affect the stability of α- or β-ketonic-monocarboxylic acids, or of α-ketonic-dicarboxylic acids.

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34. Indole Formation in *Bacterium coli commune**

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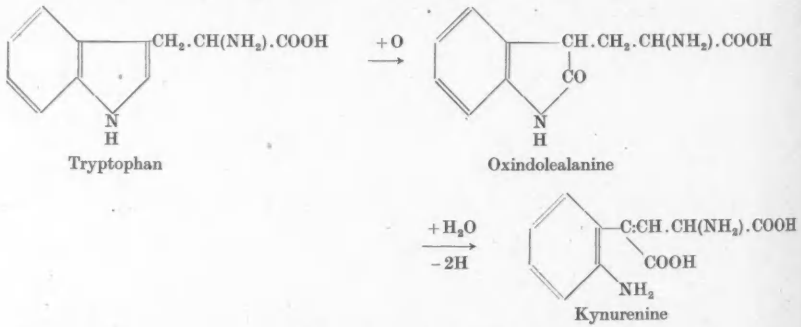
Hopkins & Cole [1903] showed 39 years ago that tryptophan is the parent substance of the indole formed by bacteria. Since then many attempts have been made to elucidate the intermediary mechanism of indole formation [see Frieber, 1922; Saito, 1933; Woods, 1935; Happold & Hoyle, 1935; Baker & Happold, 1940]; all these attempts have been unsuccessful: no intermediates have so far been isolated, nor has a substance related to tryptophan been shown to yield indole. Woods [1935] and Happold and his co-workers [1935; 1940] have tested the indole formation from 17 substances, among them 3-indolepyruvic acid, 3-indolelactic acid, 3-indolepropionic acid, 3-indoleacrylic acid, 3-indoleethylamine, 3-indoleglycine, 3-indolealdehyde, 3-indolecarboxylic acid. All these compounds may be excluded as intermediates.

The list of substances already tested comprises most of the compounds which might be expected to arise from tryptophan assuming that the decomposition starts in the side chain. The negative results obtained with these substances may be taken to suggest that the breakdown of tryptophan does not start in the side chain. This conclusion led us to examine a hypothesis of indole formation not involving a primary breakdown of the side chain. In forming such a hypothesis we were guided by the two following facts:

(1) In certain organisms (rabbit, *B. subtilis*) tryptophan is converted into kynurenine, a reaction which Kotake [1931] and Kotake & Otami [1933] formulate thus:

(1)

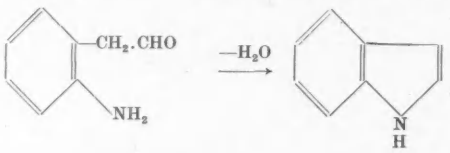


Tryptophan — Oxindolealanine — Kynurenine

In this case the decomposition of tryptophan starts in the ring, with the oxidation of the carbon atom 2.

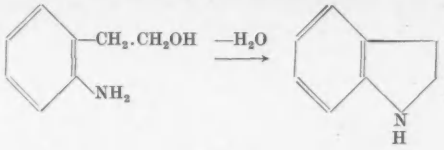
(2) Certain aniline derivatives, e.g. *o*-aminophenylacetaldehyde, or *o*-amino-β-phenylethyl alcohol readily form indole or indole derivatives *in vitro*:

(2)



[Stephen, 1925]

* A brief account of this paper appeared in *Chemistry and Industry*, 1941, **61**, 723.

(3)  [Bennett & Hafez, 1941]

On the basis of these observations we assume that the conversion of tryptophan into indole may start with an oxidation of the ring similar to reaction (1) leading, via oxindolealanine, to an *o*-aniline derivative similar to or identical with kynurenine; that the side chain of this compound is consequently broken down to *o*-aminophenylacetaldehyde which yields indole according to (2). If this hypothesis, which has already been discussed by Stephenson [1939], is correct, kynurenine and some other *o*-aniline derivatives should yield indole in *B. coli*. We have tested several compounds of this type and among them we found one substance, *o*-amino-β-phenylethanol, to react in the expected manner.

Experimental

Bacteria. The organism used in this work was a strain of *Bacterium coli commune*. It was grown at 37° on tryptic casein digest [Gladstone & Fildes, 1940] containing 2% or 3% agar. The period of incubation varied between 18 hr. and 5 days. Cells from young cultures show a high O_2 uptake when no substrate is added; in old cultures the 'endogenous' metabolism is smaller, and this is an advantage in some experiments.

When the period of incubation was completed the cells were washed twice on the centrifuge with 0·9% NaCl and then suspended in a small volume of 0·9% NaCl for storage in the refrigerator. For experiments the stock suspension was diluted with a saline medium containing 0·05 *M* phosphate buffer (*p*H 7·4) and 0·45% NaCl. Unless otherwise stated 3 ml. suspension containing about 25 mg. cells (dry weight) were measured into a conical Warburg cup and shaken at 40° under varying conditions.

Indole determination. The method of Herter & Foster [1905] as modified by Bergeim [1917] and Woods [1935] was used in most experiments. The experiment was interrupted by the addition of H_2SO_4, and an aliquot of the suspension containing about 0·1 to 0·2 mg. indole was analysed. After the addition of Na-β-naphthaquinonesulphonate and alkali to the distillate the mixture was allowed to stand 40 min. before the dye was extracted with $CHCl_3$.

The method of Fellers & Clough [1925] served to distinguish between indole and dihydroindole (indoline). Indole gives a pink colour, dihydroindole a yellow colour with *p*-dimethylaminobenzaldehyde. The reagent of Herter & Foster (Na-β-naphthaquinone sulphonate) shows no marked differences towards indole and indoline, a pink colour being formed with both compounds.

Substrates. The specimen of *o*-amino-β-phenylethanol used in this work was prepared by Bennett & Hafez [1941]. Dihydroindole was made according to the directions of the same authors. *o*-Aminocinnamic acid was obtained by reduction with $FeSO_4$ in ammoniacal solution of the respective nitro-compound. *o*-Aminoethylbenzene was prepared from *o*-nitroethylbenzene by treatment with iron filings and HCl.

Indole fermentation from o-*amino-*β*-phenylethanol*

It is shown in Table 1 that indole is readily formed from *o*-amino-β-phenylethanol. The rate of this reaction depends on the concentration of the substrate: the highest rate is observed when the concentration is about 6 mg./ml. Very high concentrations inhibit the metabolism. At the optimum concentration the rate of indole formation from *o*-amino-β-phenylethanol is more than three times higher than that from tryptophan. The O_2 uptake is increased by the addition of *o*-amino-β-phenylethanol. In the experi-

Table 1. *Indole formation from* o*-amino-β-phenylethanol and from tryptophan*

(22·5 mg. *B. coli* in 3 ml. medium; shaken in air for 6 hr. at 40°.)

Substrate added	0	2·5 mg.	5·0 mg.	10·0 mg.	20·0 mg.	40·0 mg.	0·5 mg.	1·0 mg.	10·0 mg.
		o-Amino-β-phenylethanol				Tryptophan			
mg. indole formed	0	0·104	0·389	0·636	1·25	1·17	0·155	0·306	0·416
μl. O_2 absorbed	615	714	770	915	956	790	746	931	1230

ment quoted in Table 1 the increase is considerably greater than would be expected for the reaction $NH_2.C_6H_4.CH_2.CH_2OH + \frac{1}{2}O_2 = \text{indole} + 2H_2O$, as will be seen from the last two lines of Table 1. We cannot offer a satisfactory explanation for this discrepancy. Generally the discrepancy appears to be large when the 'blank' respiration is large. In cells from old cultures where the 'blank' is small, the agreement between indole formation and extra O_2-uptake is fairly satisfactory, as seen in Table 2.

Table 2. *Indole formation from* o*-amino-β-phenylethanol in* B. coli *under varying conditions*

Conditions of culture (37°)	mg. cells used (in 3 ml. phosphate buffer)	Period of incubation at 40° hr.	μl. O_2 used by 3 ml. bacterial suspension: Blank	μl. O_2 used by 3 ml. bacterial suspension: With 10 mg. amino-phenylethanol	mg. indole: Found	mg. indole: Calc. from extra O_2 absorbed
1. Casein digest; 19 hr.	25	3	806·5	1074	1·5	2·9
2. Casein digest; 19 hr.	23·8	2	590	758	0·705	1·76
3. Casein digest; 2% glycerol; 44 hr.	26·9	3	538	770	0·295	2·43
4. Casein digest; 2% glycerol; 5 days	27·1	4	232·5	318	0·554	0·895
		6	297·5	417	1·107	1·242
5. Broth; 2% glycerol; 5 days	26·3	3	251	282	0·443	0·472
		6	420	450·5	1·005	0·320
6. Casein digest; 5 days	27·8	2	33	82·5	0·635	0·518
		4	61	169	1·253	1·13

Table 3. *Course of O_2 uptake and of indole formation in the presence of* o*-amino-β-phenylethanol*

(13 mg. *B. coli* in 3 ml. medium; 40°.)

Time hr.	No substrate μl. O_2 absorbed	4 mg. *o*-amino-β-phenylethanol: μl. O_2 absorbed	4 mg. *o*-amino-β-phenylethanol: mg. indole formed
1	90·5	135	0·085
2	157	224	0·159
4	258	361	0·333
22	700	772	1·34
30	794	820	1·48
48	927	881	1·74

The time curve of indole formation and O_2 consumption is shown in Table 3. It will be noted that the rate of O_2 uptake is increased in the earlier stages of the experiment, but it falls below that of the control in the later stages. This inhibition may be due to the indole formed; we find that a concentration of 0·5 mg. indole per ml. inhibits the 'blank' O_2 uptake of *B. coli* by 25%.

The formation of indole from *o*-amino-β-phenylethanol was confirmed by Ehrlich's test, by the nitroso-indole test [see Zoller, 1920] and by the characteristic odour.

No indole was formed when the conditions were anaerobic. The tests for dihydroindole were negative aerobically and anaerobically. Addition of dihydroindole did not cause any indole formation or O_2 consumption. It may be concluded from these observations that reaction (3) does not occur in *B. coli* and that the oxidation is the primary, the ring closure the secondary reaction when *o*-amino-β-phenylethanol is converted into indole. The intermediary compound is expected to be *o*-aminophenylacetaldehyde. This compound has not been described in the literature. Under conditions in which it was expected to arise, indole was found instead [Stephen, 1925]. The aldehyde is unstable and reacts at once to form indole (reaction 2).

We have tested two other ortho-derivatives of aniline, *o*-aminocinnamic acid and *o*-aminoethylbenzene. These did not form indole in *B. coli*.

Discussion

It may be argued that the formation of indole from *o*-amino-β-phenylethanol is not necessarily connected with the formation of indole from tryptophan. *B. coli* oxidizes ethyl alcohol and several of its homologues to the corresponding aldehydes. As the aldehyde arising from *o*-amino-β-phenylethanol is known to yield indole spontaneously, this alcohol might be expected to form indole on oxidation; the indole formation would in this case be 'accidental' and different from the mechanism of indole formation from tryptophan.

We prefer to assume that there is a link between the two indole-yielding reactions. We believe that indole is formed when, or because, a substrate yields *o*-amino-β-phenylacetaldehyde in the course of its decomposition. *o*-Amino-β-phenylethanol is not necessarily an intermediate in indole formation from tryptophan; in fact, its rather slow rate of reaction at low concentrations is against this. But the corresponding aldehyde is, in our view, an intermediate. This hypothesis has the advantage of explaining the various types of tryptophan breakdown (formation of kynurenine in the rabbit and *B. subtilis* [Kotake, 1931], of kynurenic acid in the dog, of anthranilic acid in *B. subtilis* [Kotake & Otami, 1933], of indole in *B. coli*) as modifications of one major scheme. In every case the primary step is the formation of an oxindole derivative followed by ring opening; the further breakdown of the side chain then differs from case to case. Removal of 2H atoms leads to kynurenine; a further oxidation, as suggested by Kotake [1931], leads to $NH_2.C_6H_4.C(OH):CH.CO.COOH$ and thence by ring closure to kynurenic acid; it is not difficult to draw up a series of reactions leading to *o*-amino-β-phenylacetaldehyde, though at the present this scheme would have to be entirely hypothetical.

The hypothesis also explains the failure of indole derivatives other than tryptophan to yield indole and is in accordance with the conclusion of Baker & Happold [1940] that the breakdown of tryptophan to indole requires an intact side chain.

Summary

It is shown that *o*-amino-β-phenylethanol yields indole in *B. coli*, if O_2 is available. The primary step is probably the formation of *o*-aminophenylacetaldehyde. This reacts spontaneously to form indole.

The following hypothesis accounts for the experimental observations so far available. The biological breakdown of tryptophan begins with an oxidation of carbon atom 2 of the indole ring leading to an oxindole derivative; this is followed by a ring opening yielding an *o*-aniline derivative; in the case of indole-forming organisms the side chain of the *o*-aniline compound is broken down to *o*-aminophenylacetaldehyde, whence indole arises spontaneously.

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35. The Tryptophanase-tryptophan Reaction

5. Possible Mechanisms for the Inhibition of Indole Production by Glucose in Cultures of *B. coli*

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The facts described by Happold & Hoyle [1936] and Evans *et al.* [1941] can be concisely stated as follows:

(1) In a complex medium (e.g. bouillon, casein-digest, etc.) in which the presence of glucose in sufficient amount inhibits completely the production of indole by *B. coli*, the complete tryptophanase system is absent from the cells.

(2) With a simple salt medium as used by Fildes, glucose only partly inhibits the production of indole (up to about 80%), but such inhibition can be completed by the addition of either phenylalanine or tyrosine in certain stoichiometrical relationships to the tryptophan present. This completion of the inhibition by these amino-acids is specific. The problem therefore resolves itself into two questions, (*a*) the actual mechanism of the glucose inhibition, and (*b*) the part played by these specific amino-acids.

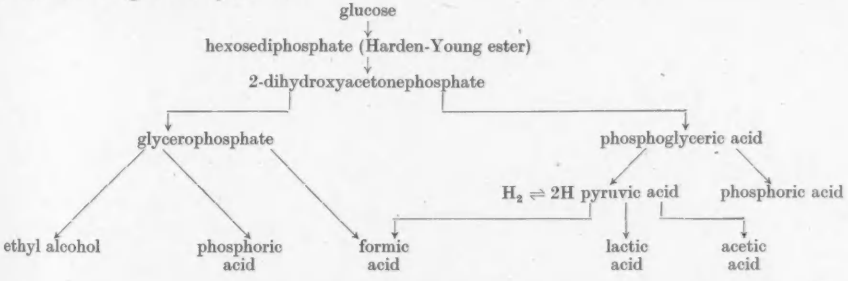
We have attempted to throw some light upon the glucose inhibition by using either:

(*a*) Complex media like bouillon or a casein-digest medium where the necessary amino-acids are present in ample quantity.

(*b*) Fildes synthetic medium together with the addition of the requisite amount of phenylalanine or tyrosine.

Effects of possible dissimilation products of glucose on indole production

Incomplete evidence supports the following scheme of the probable pathways of dissimilation of glucose by *B. coli*:



Thus, Tikka [1935] showed that when glucose was fermented with dried *B. coli* in phosphate buffer 15% of the glucose was phosphorylated after 42 hr. Hexosediphosphate and glucose were fermented by *B. coli* to give qualitatively the same fermentation products. Further, if hexosediphosphate was fermented by *B. coli* in the presence of fluoride, 95% of the reducing power disappeared, but only 54% of phosphate was liberated, thus supplying evidence for the formation of a non-reducing ester.

Stone & Werkman [1936] isolated phosphoglyceric acid from toluene-treated fermentations of *B. coli*. It is possible, however, that there may be two or more glycolytic systems present in the bacterial cell, one phosphorylating and one not.

In mammalian and yeast metabolism, the dissimilation of carbohydrate, i.e. the stages glucose → hexosediphosphate (Harden-Young ester), is a complex one involving two step-wise phosphorylations:

glucose → glucose-1-phosphate (Cori ester) → glucose-6-phosphate (Embden ester) ↓ hexosediphosphate (Harden-Young ester), fructosediphosphate

Willstätter [1940] claims to have shown that if glucose is added to a suspension of yeast cells, it disappears from solution and is built up into a polysaccharide (glycogen) inside the yeast cell. Only after this process, taking about 10 min., does fermentation (CO_2 production) begin. Whether or not this happens with *coli* cells is not known, but it appears likely from our findings [Evans *et al.* 1941], since a tryptophanase system reappears in *E. coli* cells grown on complex glucose-containing medium always after a definite lag period. This lag we assume is related to the metabolism of stored carbohydrate.

Hanes [1940] showed that glucopyranose-1-phosphate (Cori ester) added to enzymically active preparations of yeast, is partly converted into a true polysaccharide, apparently glycogen:

glycogen ⟷ glucopyranose-1-phosphate (Cori ester)
(phosphorolysis) ↓ (phosphoglucomutase)
glucopyranose-6-phosphate (Embden ester)
⇵
fructofuranose-6-phosphate
⇵
fructofuranose-1:6-diphosphate (Harden-Young ester)
⇵
2-triosephosphate

The enzymic phosphorylation of glucose in position 1 has still to be demonstrated, if it is to be accepted as an intermediary in glycogen synthesis, although there is no doubt that yeast, muscle and liver can all utilize glucose for formation of glycogen.

It seems reasonable to suppose that a similar mechanism obtains for the bacterial dissimilation of carbohydrates. In order to gain some insight into the mechanism of the inhibition due to glucose, we have prepared all the known phosphorylated-hexose intermediates to see which, if any, of these can exert a similar inhibition.

Experimental

Hexosephosphates

Cori ester (glucopyranose-1-phosphate). The white amorphous Ba salt (prepared by the method of Cori *et al.* [1937] analysed as follows:

	Found	Calc.
P [Fiske & Subbarrow, 1925]	6·7 %	6·9 %
Glucose content after acid hydrolysis	37·5 %	40·1 %
$(\alpha_D^{25°})$ of the free acid	+119	Cori *et al.* [1937] +120°

Embden ester. This ester was prepared enzymically from starch by the methods of Ostern *et al.* [1936] and Kendal & Stickland [1938]. The Embden ester so produced had a P content of 7·8 %; calc. 7·84 %. The Embden ester is not a pure substance but a mixture of glucose-fructose- and mannose-6-phosphates.

Harden-Young ester. Calcium hexosediphosphate, supplied by Bayer, was purified through the Ba salt, making use of the lower solubility of this salt in water-alcohol mixtures at 80° [cf. Harden, 1930].

Estimation of indole

Indole determinations have been made by the method of Happold & Hoyle [1934] which depends on the rapid extraction of the indole from the culture by light petroleum (B.P. 40–60°) and the addition of the Ehrlich reagent to the petroleum extract. The colours are matched in a colorimeter. The method is rapid, inexpensive and accurate to a degree not yet claimed by other methods. Nessler tubes can be used where no colorimeter is available.

Growth experiments in bouillon with the phosphorylated hexoses

The phosphorylated sugars were all added as the Na salts in concentration equivalent to the glucose used (1 %). The basal medium was ordinary bouillon. The indole, if any, was noted 8, 12 and 18 hr. after inoculation with *B. coli*.

There was no inhibition of indole formation with Harden-Young ester, glycogen, starch or maltose and no acid production during the period of growth. (A glycogenase, etc., develops by adaption on standing.) Glucose alone showed real inhibition though the Embden ester stopped indole formation up to 8 hr. and some slight inhibition may have occurred with the Cori ester, though this is less certain since growth too was retarded with this compound. Acidity developed with glucose and both monophosphoric esters but it was decidedly weaker with the Cori ester. It is clear therefore that the Harden-Young ester, maltose and the two polysaccharides do not influence the reaction because they are not absorbed by the cell with sufficient rapidity. (The late formation of a glycogenase system seems to indicate a possible slow absorption.) Knowledge of the rate of dissimilation of the monophosphoric esters, which again may be controlled by their rate of absorption, is vital to a correct assessment.

Results with synthetic media

Several experiments have been performed with synthetic media, that of Fildes and one of our own derivation. The results are qualitatively the same with both types so that a typical experiment with the former will be recorded (Table 1) and the rest of the results summarized.

Fildes's medium contained lactate as sole source of carbon except for the tryptophan essential to the reaction. We have added phenylalanine to 1 % and our tryptophan level has never exceeded 0·5 %. We have compared rates of growth and of indole formation in such media—with and without lactate ($M/2$–$M/25$) and with the substitutions of ($M/12{\cdot}5$–$M/25$) formate, acetate, pyruvate and succinate for the lactate.

The results with these substrates do not advance our knowledge but there is delay in the onset of indole production when pyruvate $M/12{\cdot}5$ is used and this effect is less marked at lesser concentrations. A similar effect with formate is less remarkable in that it can be correlated with a marked lag in the development of the logarithmic phase of growth of the organism.

Numerous experiments performed with the three hexosephosphate esters and glucose confirm that hexosediphosphate retards growth but not indole formation. The initial rate of growth with the two monophosphoric esters is generally less rapid than with the control medium and this is markedly true of the Cori ester. The rate of acid production and of disappearance from the media of these two esters is also less rapid than is that of glucose; this is especially true of the Cori ester, which in 7 days was reduced by 80 % (68·85 mg., as glucose, to 13·8 mg.) whereas a similar quantity of glucose disappeared entirely in 36 hr.

In this particularly we would draw attention to the fact that whilst Cori ester $\rightleftharpoons$ glycogen, is a demonstrable biological reaction, there is no evidence for a direct Embden ester $\rightarrow$ glycogen, change. The results can be amply accounted for by the differences in the rate of utilization of the carbohydrate derivatives involved.

Table 1. *Growth experiments in Fildes's synthetic medium with hexosephosphates*

Basal medium: Fildes's synthetic medium, 20 ml.; tryptophan, 0·1 ml. (0·1 g./20 ml.); phenylalanine, 0·5 ml. (0·2 g./20 ml.); glucose and substrates added in 1 % equivalent solution; indole determinations on 2 ml. of culture.

Time ...	3 hr.		9 hr.		12 hr.		18 hr.		31 hr.	
Medium	Indole μg.	Growth	Indole μg.	Growth	Indole μg.	Growth	Indole μg.	Growth	Indole μg.	Growth
+Lactate *M*/2	½	–	2	+	9	+ + +	14		22	
Cori ester	Nil	–	½	±	10	±	14	+ +	25	
Embden ester	Nil	–	1	+ +	1	+ + +	10		15	
Glucose	1	–	1	+ +	1	+ + +	Trace		Nil	
Control	1	–	4	+	12	+ +	18		30	

– =no visible growth.

The effect of other carbohydrates on indole production

Peptone water was used, the sugar to be tested was added to 1 % concentration and the solution sterilized by filtration. Observations were recorded after 24 hr. growth; different results are obtained with some carbohydrates which are not fermented here when they are subjected to prolonged incubation, but this involves adaptation. The results are summarized under three headings.

I. *No acid production or inhibition of indole formation* was obtained with: glycogen, starch, dextrin, saccharose, dulcitol, salicin, inulin, glycerol and potassium saccharate.

II. *Acid production and complete inhibition of indole formation* were obtained with: arabinose, lactose, glucose, fructose, mannitol and potassium *d*-gluconate.

III. *Acid production without marked inhibition of indole production* was obtained with: *d*-ribose, rhamnose, glucosamine HCl (20 μg. indole after 7 hr. growth = value for control medium), xylose, sorbitol and galactose (15 μg. indole at 7 hr.) and mannose (8 μg. indole).

Glyceraldehyde in concentrations down to *M*/1000 inhibited all growth in this medium but it should be noted that the effectiveness of the glyceraldehyde is not permanent and that complete inhibition can only be guaranteed if inoculation is made from a suspension of washed cells.

The third group of sugars is particularly interesting since we have an intense fermentation without inhibition of the indole production (mannose is at least as rapidly fermented as glucose). A common structural clue to this difference in action between groups II and III is not apparent. This difference in action between sugars in groups II and III is further support to the evidence previously presented [Happold & Hoyle, 1936] that the failure of indole production in glucose peptone water cannot be ascribed to competitive removal from the medium, by the glycolytic system, of a coenzyme factor essential to the tryptophanase system.

The effect of inhibitors of carbohydrate metabolism upon the production of indole

Fildes's [1938] medium and bouillon, both with and without glucose (1 %), were used. Na_2SO_3 and NaF (*M*/100) did not inhibit indole production in the control media nor prevent the inhibition of indole production in the glucose cultures. Phloridzin was equally ineffective, whilst sodium iodoacetate prevented all growth. The results with glyceraldehyde are presented in Table 2. The glyceraldehyde does not affect the *p*H of the bouillon cultures. It will be observed that glyceraldehyde prevents growth of the organisms at *M*/100 concentration, at a lesser concentration it inhibits indole formation without affecting growth and at still weaker concentrations is without any action. At 72, but not at 48, hr. the bouillon + glyceraldehyde (*M*/500) shows indole formation. These

Table 2. *Effect of glyceraldehyde*

Medium	After 24 hr. Indole production	Growth
Bouillon	+ + + (50 μg./2 ml.)	Good
Bouillon + glyceraldehyde, *M*/1000	+ + +	,,
,, , *M*/500	Nil	,,
,, , *M*/100	Nil	Nil
Glucose bouillon + glyceraldehyde, *M*/1000	Nil	Good
,, , *M*/1000	Nil	Good

concentrations may be variable since Mendel *et al.* [1931] have shown that the glyceraldehyde effect is inhibited by small traces of pyruvic acid, and Needham & Lehmann [1937] report a similar effect with hexosediphosphate; the presence of some such reactant in the bouillon is the most likely explanation of the decreased activity in this medium as compared to peptone water. It should be noted that glyceraldehyde has been postulated as an intermediate in non-phosphorylating glucolysis.

Table 3. *Action of inhibitors of glucolysis on the appearance of indole when tryptophan is acted upon by suspensions of* B. coli *grown on casein digest-glucose agar*

Washed cells from 24 hr. culture (3 Roux bottles) were suspended in 70 ml. ($\frac{1}{2}$ vol. 0·85 % NaCl, $\frac{1}{2}$ vol. *M*/15 phosphate buffer). There was no indole in the washings.

10 ml. of suspension + 10 ml. NaCl-phosphate buffer + 1 ml. inhibitor + 1 mg. tryptophan in 0·2 ml. were mixed in each flask and incubated at 37°. Indole determinations were made on 2 ml. samples, but are represented as the total amounts which would be present in the intact flask at the time of sampling.

Addition	μg. indole 0 min.	30 min.	60 min.	120 min.
None	Nil	21	105	525
Phloridzin, *M*/200	,,	Nil	21	472
NaF, *M*/50	,,	Trace 10	52	525
Iodoacetate, *M*/33	,,	Nil	Nil	Nil
dl-Glyceraldehyde, *M*/100	,,	10	21	262
Glucose, 0·1 %	,,	Nil	Nil	Trace
Toluene	,,	,,	,,	Nil

It may be that there is some slight delay in the recovery of tryptophanase action by the cells in the presence of phloridzin and NaF. The results with *dl*-glyceraldehyde and sodium iodoacetate are more definite.

A more extensive series with glyceraldehyde + 0·5 mg. tryptophan gave the following:

	μg. indole formed after 0 min.	30 min.	90 min.	150 min.	210 min.
Cells + glyceraldehyde, *M*/100	Nil	Nil	Nil	<1	8
,, , *M*/1000	,,	,,	20	150	220
Cells alone	,,	,,	22	182	240

The effect with glyceraldehyde is thus definite but not permanent as with toluene. When active tryptophanase suspensions were incubated for 10 min. with *M*/100 glyceraldehyde and then tested against tryptophan there was some loss of activity (11–15 %); when subjected to the action of *M*/20 glyceraldehyde the preparation was inactivated. There is thus no guarantee that the increased delay in indole production by cells grown in the presence of glucose and treated with glyceraldehyde is not due to direct action upon the tryptophanase system. That the iodoacetate effect is also non-permanent is shown

by the following results. The results with cells grown in the absence of glucose show that iodoacetate has little direct action upon the tryptophanase system.

	μg. indole formed after			
	0 min.	30 min.	50 min.	3 hr.
Normal cells + 1 mg. tryptophan	105	315	472	—
" + *M*/33 iodoacetate	84	265	420	—
Glucose cells + 1 mg. tryptophan	Nil	Nil	105	420
" + *M*/33 iodoacetate	"	"	Nil	21

When the concentration of iodoacetate is further reduced to *M*/333 indole formation is quickened and enhanced.

The effect of K^+ on tryptophanase regeneration in glucose agar cells

Pulver & Verzar [1940] suggest that the yeast cell respiring in the presence of glucose and salts first builds up the glucose into a polysaccharide (probably glycogen) before metabolizing the carbohydrate. The first phase it is suggested is associated with an absorption of K^+ from the medium, whilst during active fermentation K^+ is released from the cells into the surrounding medium.

The lag period in indole production which occurs when the washed cells of *B. coli*, grown in glucose agar, are placed in contact with tryptophan, had all the appearance of an intimate relationship with the breakdown of stored carbohydrate. Consequently tests were made on the effect of K and Na ions on the rate of recovery of indole production.

Cells grown on glucose bouillon agar for 18 hr. were washed off with sterile water, resuspended in *M*/20 sodium phosphate buffer and divided. One half was diluted 1/9 with 0·5 % NaCl (NaCl concentration 0·45 %, Na phosphate *M*/200) and the other half with 0·5 % KCl (KCl 0·45 %, Na phosphate *M*/200). They were again washed in the centrifuge and the deposited cells suspended again in these mixtures to which was added tryptophan so that there would be 100 μg. to every 5 ml. They were incubated at 37°. No indole was produced in either series up to 90 min. The results were as follows:

	Indole, μg./5 ml.	
min.	0·45 % KCl system	0·45 % NaCl system
90	Nil	Nil
120	25	"
135	50	$\frac{1}{2}$
180	53	6

This experiment, frequently repeated, always showed indole formation in the K^+ series before the Na^+ series. These results give indirect evidence that the breakdown of reserve carbohydrate precedes the reactivations of the tryptophanase system.

With the assistance of three of our senior students (J. Dawson, F. M. Parsons and W. Whitaker) who have been working under the supervision of one of us (F. C. H.) it has been possible to carry out a more extensive series of determinations in which complete curves were obtained. The molar and osmotic relationships were varied and concentrations reduced. Suspensions have been washed with the salt solutions finally used.

(i) Sodium phosphate buffer, Na concentration = 47·7 mg./200 ml.

(ii) Sodium phosphate buffer, Na = 23 mg./ml., + equal vol. of potassium phosphate buffer, K = 21 mg./ml.

No NaCl or KCl was included. The tests were carried out in a series of 125 ml. Erlenmeyer pyrex flasks and each contained 12 ml. buffer, 2 ml. tryptophan (1/10,000) and

1 ml. of the bacterial suspension. The following values, typical of others were obtained:

min.	μg. indole in Na system	μg. indole in Na + K system
20 & 40	Nil	Nil
60	1	5
80	1·5	13
100	3	13·5
120	7	38·5
140	8	53·0
160	45	63·5
180	73	61·5
200	73	63

The increased lag in the absence of K^+ is apparent in the slower rate of acceleration in indole production after its commencement. These facts present further indirect evidence in support of the hypothesis that the metabolism of stored carbohydrate prevents the functioning of the adaptive tryptophanase system in cells grown in the presence of complex media containing glucose.

Discussion

The results presented are unavoidably incomplete in some directions. Studies on carbohydrate storage have made some progress and will show that the lag in tryptophanase recovery by the *coli* cells grown on a glucose nutrient medium is associated with the breakdown of stored carbohydrate [J. Dawson & F. C. Happold, unpublished]. This point is already indicated in the results presented in this paper concerning the effect of K ions on the rate of recovery of tryptophanase.

Needham & Lehmann [1937] have shown that freshly prepared glyceraldehyde inhibits glycolysis of glycogen in muscle extract in the same way as glucose does, the inhibition occurring at the stage glycogen $\rightleftharpoons$ Cori ester.

Stickland [1941] has shown that inhibition of the conversion of glucose into Cori ester occurs with the monomeric form. The preparations of glyceraldehyde used in our experiments were in the dimeric form, or predominantly so, and the results here reported might be explained by the inability of the tryptophanase system to function while glycolysis is occurring in the cell.

Iodoacetate it is claimed inhibits the reaction triosephosphate $\rightleftharpoons$ 3-phosphoglycerate, i.e. the first oxidation process in the glycolytic system: from the present experiments it would appear that interference in the glycolysis at this stage also blocks the recovery of tryptophanase action, neither is this recovery hastened in the presence of excess of cozymase. According to Adler *et al.* [1938] the action of the iodoacetate is upon the triose dehydrogenase itself.

The ineffectiveness of fluoride and sulphate as well as the results of experiments using lactate, pyruvate, formate etc., as energy sources in growth experiments, indicates quite definitely that the secondary oxidation system in glucolysis is without action upon the inhibition of tryptophanase action by the organism.

The inhibitory action upon the growth of *B. coli* of the presence of the hexosephosphates was surprising as was the fact that whilst the two monophosphates were fermented the rate was slower than with glucose, this was especially true of the Cori ester. The inhibition of indole production in cultures was less marked with these esters than with glucose, but until something is known of their rate of absorption as compared with that of the unphosphorylated carbohydrate, the results shed no new light upon the problem.

We must again stress that the tryptophanase system is adaptive and that the previous nutritional history of the cell during growth determines its activity. The combined action of the glucose and phenylalanine is only effective whilst the cells are still growing, they are without action upon the preformed tryptophanase system of suspensions which are still viable.

We must reject the suggestion that the system is rendered inoperative because of an increase in H-ion concentration during the fermentation of the carbohydrate, in view of the results obtained with mannose and the sugars grouped with it, and because of repeated failure over a period of years to obtain any tryptophanase action from preparations from cells grown in complex media containing glucose.

Summary

Glycogen, starch, dextrin, saccharose, dulcitol, salicin, inulin, potassium saccharate and hexosediphosphate are not fermented in 24 hr. cultures of *B. coli*, nor do they affect the production of indole when tryptophan is present in the medium.

Complete inhibition of indole production is obtained with arabinose, lactose, glucose, fructose, mannitol and potassium *d*-gluconate and some with Embden ester. The position with the Cori ester is less easy to assess since it is utilized less rapidly.

Acid production without marked inhibition of indole production was obtained with rhamnose, glucosamine hydrochloride, xylose, sorbitol, galactose, *d*-ribose and mannose.

Glyceraldehyde in freshly prepared solutions inhibits growth in some media in concentrations of *M*/1000. It continues to inhibit indole production at concentrations at which it does not affect growth. *M*/100 glyceraldehyde reduces the activity of the system tryptophanase-tryptophan → indole and at *M*/20 completely stops the reaction.

Iodoacetate (*M*/33) retards the recovery of tryptophanase action by cells grown on glucose-casein-digest agar, its effect upon preformed tryptophanase is slight. Other inhibitors of glycolysis are without action upon the rate of recovery of tryptophanase action by such suspensions. This recovery is delayed in the absence of K ions.

It is concluded that the inhibition of indole production by cells grown in the presence of glucose and phenylalanine is maintained by the metabolism of stored carbohydrate and that such inhibition may be operative down to the stage triosephosphate → phosphoglycerate.

Our thanks are due to Prof. A. R. Todd for the gift of *d*-ribose and to the Medical Research Council for a grant to one of us.

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36. A Critical Analysis of the Tissue Slice Method in Manometric Experiments

Effect of Variations in O_2- and CO_2-tension

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Tissue slices are very frequently used in manometric experiments for the determination of a variety of metabolic reactions of tissues. The interpretation of such experiments as physiological, i.e. as giving a true picture of the actual happenings in the intact organ or in the body, has been widespread and often unreserved.

In this paper an attempt is made to analyse the conditions prevailing in experiments with tissue slices *in vitro* and to assess how far one is justified in attributing physiological significance to such experiments. The use of minced tissue as compared with tissue slices is also examined from the same point of view.

1. EXPERIMENTS IN ABSENCE OF CO_2

Method

The experiments described in the first part of the paper were carried out at 38·5° using the ordinary (open) Warburg manometer with conical vessels. The fluid used was Ringer-phosphate (3·0 ml.). The inner cup contained 0·3 ml. 10 % KOH and a slightly protruding filter paper (Whatman's no. 40).

Substances to be added during the course of the experiment were tipped into the main part of the vessel from the side bulb, and were dissolved in Ringer-phosphate solution with adjustment of *p*H to neutrality where necessary.

If the gas had to be changed during the experiment (O_2 after N_2) the gas, 300–400 ml., was passed through the vessels and escaped through their stoppers while the vessels were shaken in the water bath. The gas was taken from a set of aspirator bottles in the same water bath and, therefore, had exactly the required temperature, thus allowing a new reading almost immediately after the vessels had been re-filled with the gas. The tissues used were obtained from the mouse and comprised slices of kidney and muscle, intact portions of diaphragm, or minced diaphragm or abdominal muscles.

Results

A. *Tissue slices and O_2-tension*

It was shown by Warburg [1923] that slices, in order to be evenly provided with O_2, must be thinner than $4{\cdot}7 \times 10^{-2}$ cm. in pure O_2 and thinner than $2{\cdot}1 \times 10^{-2}$ cm. in air. Since the difficulty of preparing slices is greater the thinner they are required to be, the respiration of slices was generally measured by him in pure O_2.

This method was generally adopted by other workers and all aerobic experiments on tissue slices have been carried out either in 100 % O_2 or in a mixture of 95 % O_2 + 5 % CO_2.

It has been shown previously [Laser, 1937] that although the magnitude of respiration of a variety of tissues in Ringer-bicarbonate and in presence of glucose was to a wide extent independent of the O_2-tension, the aerobic glycolysis on the contrary appeared or was increased under low O_2-tension. Stress was laid on the point that experiments carried out in 5 % O_2 represent more the normal O_2-tension of the body than experiments with 100 % O_2.

The rate of O_2-uptake of kidney slices without addition of substrate and in presence of sodium lactate ($M/50$) in 100 % O_2 is shown in Fig. 1. The shape of both curves is characteristic and always more or less the same. Sometimes, however, O_2-uptake in presence of lactate does not remain steady so long as 90 min., but falls off earlier. In absence of any added substrate (curve I) O_2 uptake always decreases rapidly and regularly at the beginning, the relative decrease however becomes less marked with time, i.e. the curve representing the rate of O_2-uptake flattens out. This slope of the O_2-uptake curve may be due to the following reasons.

(1) Tissue slices represent a heterogeneous population of cells, some of which being more differentiated than others may therefore be more labile and die at the beginning of the experiment. Generally however elimination of tissue cells from respiration through death cannot be made responsible for the particular slope of the curve for two reasons. First, if the number of dying cells increases at a uniform rate the curve showing the O_2-uptake of the remaining cells should decline linearly. Secondly, if the rate of dying cells increases with time, as would seem to be more likely, the curve should show an increasing fall with time. The actual shape of the curve does not agree with either of these assumptions.

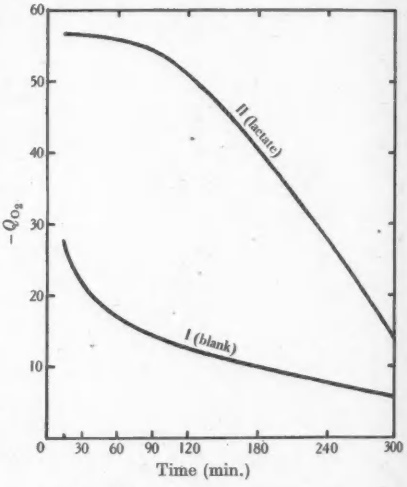

Fig. 1. Mouse kidney slices. Q_{O_2} without addition of substrate (I) and with sodium lactate ($M/50$) (II).

(2) The diffusion out of the cells into the surrounding medium of some substances which cannot be replaced by the cells but which are normally provided within the body. That some such diffusion takes place is obvious from the appearance of the medium even after experiments of comparatively short duration. These substances however have no relation to the rate of the O_2-uptake. If different amounts of tissue are suspended in the same amount of fluid, their rate of respiration is absolutely the same over long periods. It can be proved, however, that some oxidizable substances diffuse out, because fresh tissue slices shaken in a medium in which other slices have previously been kept for some hours start with a higher rate of respiration than their controls, but the relative shape of the curve is not changed.

That diffusion alone is not responsible for the decrease in the rate of O_2-uptake is shown also by the difference in relative decline of tissue respiration in presence and in absence of substrate. The rate of diffusion should be the same in both cases, and both curves, although lying at different levels should run parallel. This is however not the case, as shown by the experiment summarized in Fig. 1. Among other metabolic reactions, the aerobic acid production, however, is affected by diffusion of a substance out of the cells. This explains the results of Elliott & Baker [1935] who have shown that aerobic acid production of retina decreases in proportion to the increase in the amount of tissue suspended in a given volume of fluid.

(3) It is also conceivable that a substance essential for normal cellular metabolism may be used up rapidly at the beginning of the experiment in absence of added substrate. Experimental evidence for this assumption is summarized in Fig. 2. Here the Q_{O_2} of kidney slices is plotted as function of the O_2-tension at different times of the experiment. Q_{O_2} at the beginning of the experiment was gained by extrapolation from the first two

readings at 10 and 20 min. It will be seen that only for the first 7½ min. was the Q_{O_2} higher in 100 % O_2 than in 20 % O_2. Later the value of Q_{O_2} in 100 % O_2 decreased more rapidly than at lower O_2-tension so that after 90 min. it is actually smaller than in 10 % O_2. If each initial rate at the different O_2-tensions is taken as 100, then the % decline of Q_{O_2} increases with increasing O_2-tension (Fig. 3). This observation suggests that some substance is oxidized at the beginning of the experiment *in vitro* and that this oxidation is more rapid in 100 % O_2 than at lower tensions.

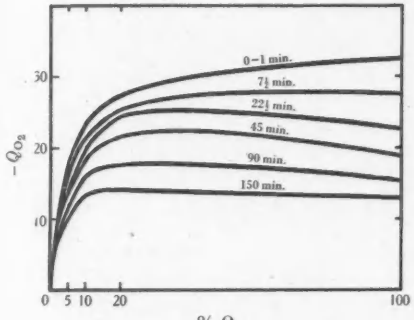

Fig. 2.

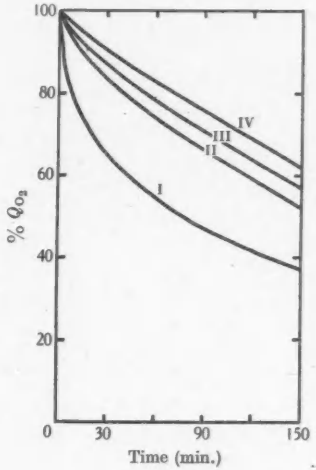

Fig. 3.

Fig. 2. Mouse kidney slices. Q_{O_2} as function of the O_2-tension at different periods of the experiment.

Fig. 3. Mouse kidney slices. Percentage decline of Q_{O_2} with time at varying O_2-tensions. Each *initial* value at the respective O_2-tension = 100. I = 100 % O_2; II = 20 % O_2; III = 10 % O_2; IV = 5 % O_2.

Q_{O_2} curves as function of the O_2-tension have been obtained with succinate and lactate as substrates in the same way as without substrate. To compare the three sets of curves they have been plotted in a different way (Figs. 4 and 5) to show the relative changes of Q_{O_2} which occur with time at varying O_2-tensions. Fig. 4 shows their relation at the beginning of the experiment and Fig. 5 the state at $t = 45$ min. In each case the respective Q_{O_2} in 100 % O_2 has been taken as 100. The different curves, therefore, represent the values of respiration under different O_2-tensions expressed as percentage of their respective Q_{O_2} in 100 % O_2.

At the beginning of the experiment (Fig. 4) the rate of succinic oxidation in 10 % O_2 is already 93 % of that in 100 % O_2, while the rate of lactate oxidation in 20 % O_2 is only 75 % of that in 100 % O_2. The curve representing the oxidation rate of the substances which are present in tissue slices and are being oxidized if no substrate has been added lies somewhere between the two former values.

Fig. 5 describes the corresponding oxidation rates for the period 30–60 min., i.e. it shows the state at $t = 45$ min. The curve involving the succinic system has remained unaltered while the two other curves have changed in opposite directions. The rate of lactate oxidation has now still more declined at lower O_2-tensions, while tissue slices without added substrate show actually a higher rate of respiration between 10 and 20 % O_2 than at 100 % O_2.

The O_2 affinity is a property of the enzyme which combines with O_2. It is conceivable that in the aerobic oxidation of lactate two independent systems may be involved: the cytochrome system and another system which has a much lower affinity for O_2.

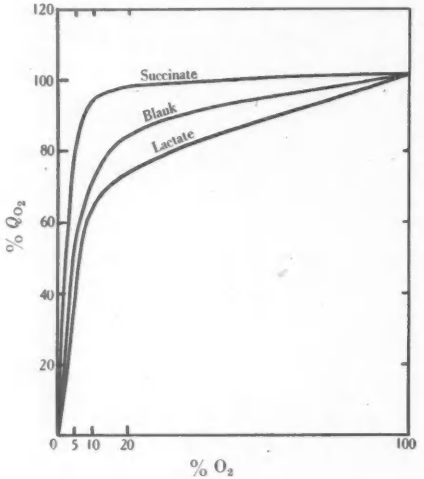

Fig. 4.

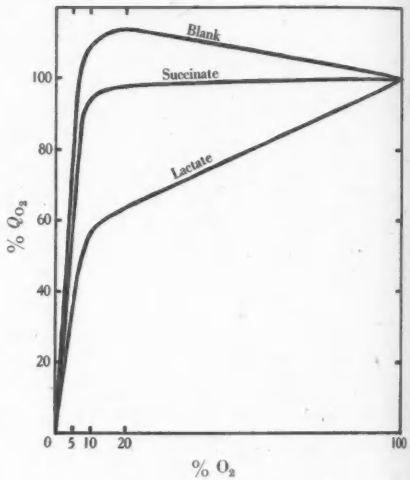

Fig. 5.

Fig. 4. Mouse kidney slices. Q_{O_2} at different O_2-tensions expressed as percentage of the Q_{O_2} in 100 % O_2 (=100) without addition of substrate and with sodium succinate and lactate ($M/50$) at the beginning of the experiment.

Fig. 5. Mouse kidney slices. Same as Fig. 4 for the period 30–60 min., i.e. at $t=45$ min.

B. *The addition of substrates and time factor*

The O_2-uptake of tissue slices was also measured when different substrates were added after a preliminary period of respiration in absence of any added substrate. Several substrates and combinations of them have been tried such as glucose, sodium lactate, pyruvate, fumarate, citrate, succinate and boiled yeast extract.

The results of these experiments fall into three groups: (1) the O_2-uptake by the tissue after the addition of the substrate reaches the same rate which it has in presence of the same substrate added at the beginning of the experiment; (2) it reaches the rate of O_2-uptake of the control sample at the time of adding the substrate; (3) it remains lower than the rate of O_2-uptake of the control at the time of adding the substrate.

To the first category belongs only sodium succinate, i.e. the system catalysing its oxidation is not affected either by diffusion away or oxidation of one of the respiratory components during the time that tissue slices respire in absence of succinate (Fig. 6). To the second category belong sodium fumarate (Fig. 7) and pyruvate, citrate and glucose, additions of which give similar curves to those shown in Fig. 7. The activity of the systems catalysing their oxidation is noticeably weakened in tissue slices which have been kept for some time without the respective substrates; their activity declined to the same extent as that of the control samples in presence of substrates. Both oxidation rates are therefore equal after the substrates have been added. In the case of some of these substances however (fumarate and citrate) O_2-uptake in presence of the respective substrates had already fallen off so much by the time the substrates were added to the slices devoid of them that it needed only a slight rise in the oxidation rate

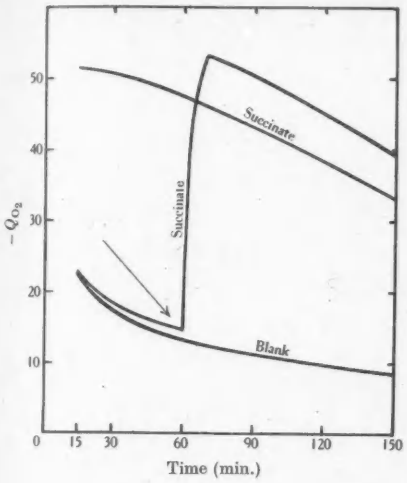

Fig. 6.

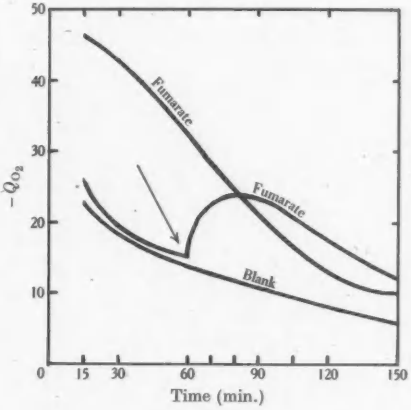

Fig. 7.

Fig. 6. Mouse kidney slices. Q_{O_2} on addition of sodium succinate ($M/50$) after 60 min. incubation in 100 % O_2 without added substrate, and two controls, i.e. without added substrate throughout (blank) and with succinate present from the beginning.

Fig. 7. Mouse kidney slices. Same as Fig. 6 with fumarate ($M/50$) as substrate.

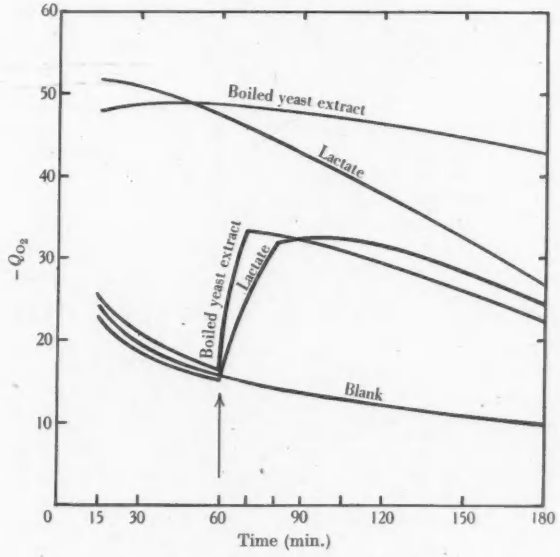

Fig. 8. Mouse kidney slices, Same as Fig. 6 with lactate ($M/50$) and with 0·2 ml. boiled yeast extract as substrates.

to reach that level. To the third category belong sodium lactate and boiled yeast extract (Fig. 8). The activity of the catalytic systems involved in these oxidations has been considerably reduced during the preliminary stage. On addition of these substrates, although the rate of oxidation is increased it does not reach the rate shown by the control slices at the same time. It is remarkable that the addition of boiled yeast extract did not raise the rate of O_2-uptake more than described above, since when added at the beginning of the experiment it not only raises the O_2-uptake considerably but keeps it at a steady rate for a considerable period of time (5 hr.).

Considering that no other substrates have been found to produce this effect, it seems safe to assume that this particular action of boiled yeast extract is not due to the presence of substrates in the extract but to an additional factor. It is not the presence of coenzyme I which causes respiration to remain steady because the addition of coenzyme either alone or in combination with sodium succinate, fumarate and lactate did not stabilize the rate of O_2-uptake. In this respect the tissue slices examined differ from muscle mince, the respiration of which can be stabilized by the addition of fumarate, especially in presence of small amount of tissue extracts [Stare & Baumann, 1937]. That a substance other than substrate or coenzyme is lost by the slices, owing to its oxidation could be proved by the following experiments.

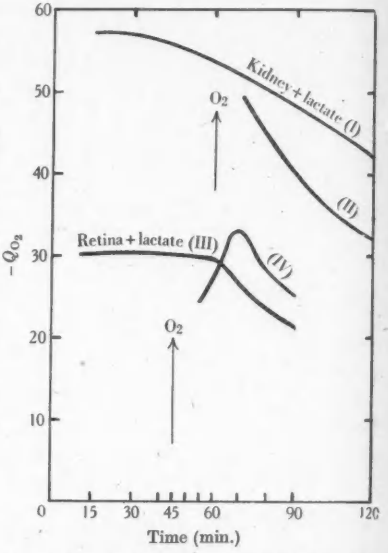

Fig. 9. Mouse kidney slices: I and II. Pig retina: III and IV. I and III: Q_{O_2} in presence of lactate in 100 % O_2. II and IV: preliminary incubation in presence of lactate for 45 and 60 min. respectively in N_2, when 100 % O_2 was admitted. Note higher rate of O_2-uptake after admission of O_2 than after incubation in O_2 and addition of substrate after 60 min. (Fig. 8).

Kidney slices and retina were shaken in the manometer vessels 45–60 min. in presence of sodium lactate in N_2, and at the end of this period O_2 was passed through the vessels (see Methods). It was then found that the O_2-uptake in these experiments was higher than that of tissues to which substrate was added after they had been kept in 100 % O_2 for the same length of time. In most cases under these conditions the type 3 of recovery of respiration due to the addition of substrate reverted to the type 2, e.g. it reached the rate of the O_2-uptake of the control at the time t' (Fig. 9).

C. *O_2 uptake of minced and ground tissues*

Minced tissues are capable of utilizing molecular O_2 without addition of any substrate and of oxidizing a number of substrates. In most cases the Q_{O_2} of minced tissue is considerably smaller than that of tissue slices. Their O_2-uptake is of about the same order of magnitude only if both are measured in phosphate buffer solution. This, however, is not the optimum medium for tissue slices, which require a balanced saline-phosphate solution containing Ca^{++} and Mg^{++} in addition to phosphates. The requirement of minced muscle in this respect has been described differently by different authors. While Stare & Baumann [1937] obtained optimal response on addition of fumarate in phosphate-Ringer solution, Meyerhof [1919; 1930] uses phosphate buffer solution as do Krebs & Eggleston [1938], who state that Ca^{++} and Mg^{++} have a depressing effect on the O_2-uptake of minced muscle.

The discrepancy between slices and mince is also very great in their ability to oxidize added substrates. I have confirmed the results of Krebs & Eggleston [1938] that many substances which are oxidized by tissue slices are not oxidized by mince.

But even with those substances which are oxidized by slices (or intact tissues) and mince there exists a great difference in the rates of their oxidation. Thus sodium succinate increases the O_2-uptake of intact muscle (diaphragm) by 100–150 % but that of muscle pulp by 700–800 %.

The respiratory activity of mince depends furthermore upon the degree of destruction of cellular structure. If the structure of the cells is thoroughly destroyed by grinding (with or without sand) the respiration becomes very low, in the case of muscle reaching only 4 % of that of the intact muscle. This very small oxidation has obviously no longer much in common with the normal mechanism of respiration, because such a strong respiratory inhibitor as HCN affects very little or not at all the O_2-uptake of ground muscle in absence of substrate or in presence of sodium lactate (Table 1). The oxidation

Table 1. *Percentage inhibition of O_2-uptake of intact diaphragm and ground skeletal muscle of the mouse*

Ringer-phosphate, 100 % O_2.

	No substrate added		+Lactate, *M*/50		+Succinate, *M*/50	
Inhibitor	Intact	Ground	Intact	Ground	Intact	Ground
Pyrophosphate, *M*/100	0	40	0	0	10–15	68
HCN, *M*/1000	80	0–20	80	0	80	60
Sodium azide, *M*/500	70	0	0–10	0	12–18	37

of sodium succinate, however, is inhibited by HCN in ground muscle only slightly less than in intact muscle. It must, however, be remembered that ground muscle oxidizes sodium succinate at a much higher rate than intact muscle. As regards other respiratory inhibitors, Dixon & Elliott [1929] showed that pyrophosphate acts as inhibitor of respiration of muscle pulp; Leloir & Dixon [1937] have also demonstrated that pyrophosphate acts as a specific inhibitor of succinic dehydrogenase. The action of pyrophosphate on respiration of intact and ground muscle (Table 1) is in agreement with these earlier reports. It may be added, however, that acting on intact muscle, *M*/50 pyrophosphate *increases* the O_2-uptake by 50–100 %. NaN_3, which inhibits strongly respiration of yeast cells in an acid medium [Keilin, 1937], was tested. In experiments with kidney slices it was found that *M*/500 NaN_3 inhibited respiration by 60–80 % at *p*H 6·8 and by 40–60 % at *p*H 7·3. The change in *p*H did not itself affect the rate of respiration of kidney slices with or without addition of substrate (glucose). The respiration of intact muscle without addition of substrate was 70 % inhibited by NaN_3. In presence of lactate or succinate it was inhibited by 10–20 % only. The O_2-uptake by ground muscle alone or in presence of lactate was not inhibited at all by sodium azide, but with sodium succinate the inhibition was greater in pulp than in the intact muscle. These muscle experiments were all at *p*H 7·3.

D. *The validity of measurements of* R.Q.

The R.Q. represents the sum of many complex reactions. Since, as has been shown above, considerable relative changes of the metabolism of tissue slices occur with time it becomes obvious that the R.Q. can only be determined with any expectation of physiological significance in experiments of comparatively short duration.

Of the commonly used manometric methods for the determination of the R.Q. (Dickens & Šimer [1930; 1931], Dixon & Keilin [1933], Sumerson [1939]), the method of Dickens & Šimer requires by far the longest time. Some of the experiments recorded by Dickens & Šimer took as long as 3–5 hr. This very long duration of the experiments

perhaps explains why their results have not been confirmed by Elliott & Baker [1935]. I too found for rat retina, with the method of Dixon & Keilin in a 45 min. measurement and in presence of glucose, an R.Q. of 0·8 instead of 1·0 as described by Dickens & Šimer.

Discussion

It has been shown in Part 1, above, that several distinct processes occurring in tissue slices show marked differences in their dependence on the O_2-tension at different periods of the experiment. Thus the respiration in absence of added substrate falls off much more rapidly in 100 % O_2 than at lower O_2-tensions. After 60 min. respiration under the respective O_2-tensions the rate of respiration is actually higher at an O_2-tension between 10 and 20 % than in 100 % O_2.

Secondly, the additional oxidations, as of sodium lactate, show also a change of their O_2-dependence with time, e.g. the additional O_2-uptake at the beginning of the experiment in 20 % O_2 is 66 % of that in 100 % O_2. After 60 min. under the respective O_2-tensions the additional O_2-uptake in 20 % is only 27 % of that in 100 % O_2. The dependence of the lactate oxidizing system on the O_2-tension therefore has changed with time in the reverse direction from the oxidation of those substances which are oxidized in absence of any added substrate.

While these are only quantitative differences in the way in which the magnitude of respiration and of additional oxidation differs with the O_2-tension and changes for each oxidative system differently with time, it cannot be excluded that qualitative changes may also occur similarly. In fact it is conceivable that under the influence of the O_2-hypertension (100 %) usually applied *in vitro*, some reactions are set in motion which under the conditions of the low O_2-tension within the body do not normally occur at all.

The next point to be considered is the stability of the different enzymic systems in tissue slices *in vitro*. The usually sharp decline of the O_2-uptake (except when boiled yeast extract has been added) after a variable period cannot be attributed to progressive death of tissue cells from considerations of the curve of O_2-uptake of tissue slices without addition of substrates. It must be attributed either to exhaustion or to an irreversible oxidation or to diffusion of some factor out of the system. Probably all three factors act together. It has been proved, however, that oxidation of some factor is at least partly responsible for the rapid decline, because in the case of sodium lactate oxidation starts at a higher rate after preliminary incubation in N_2 than the oxidation rate of slices which were kept for the same period in O_2. The instability of the different enzymic systems in absence of their specific substrates is also proved by the fact that, with the exception of sodium succinate, the rate of O_2-uptake never reaches that of the control in presence of the substrate at the beginning of the experiment, if the substrate is added at a later time. With sodium lactate and boiled yeast extract it does not even reach the rate of O_2-uptake of the control at the time of adding the substrate.

This fact raises the further question of how different systems in tissue slices are affected by the treatment which preceded the actual experiment, e.g. preparation of slices, time which elapsed before the beginning of the experiment, and the medium used during this period.

Mince shows still greater anomalies in respect of its suitability for the measurement of oxidative metabolism. The optimum oxidative power of tissue cells is dependent on their intact structure because this guarantees the biochemical pattern of the cell and the mutual accessibility and relative concentrations of the different catalysts. The discrepancy between slices and pulp in their ionic requirements necessitated the introduction of two subsidiary hypotheses to explain the conditions in mince: (1) the diffusion of catalysts and coenzymes out of minced cells; and (2) the effect of Ca^{++} and Mg^{++} which may reach intracellular enzymes of mince to which they have normally no access. The inability of muscle mince to oxidize many substances which are oxidized by the intact

muscle required two more assumptions: (1) the possibility that the enzymes in mince are already saturated with the substrates, or (2) that the activity of the enzymes concerned in mince is insignificant [Krebs & Eggleston, 1938].

Furthermore, the degree of cell destruction has to be taken into account, to assess how far the oxidative processes are still to be considered as normal. If the tissue cells are thoroughly destroyed by grinding, their respiration not only decreases very greatly (in the case of muscle to 4% of that of the intact muscle) but it changes qualitatively as is shown by the fact that this small residual respiration is very little or not at all affected by HCN or NaN_3.

The conditions prevailing in mince become still more complicated when several organic and inorganic substances are allowed to act on it simultaneously and when the reactions occur in 100% O_2, which alters to an unknown degree the relative velocity of several enzymic processes. The experiments become therefore dependent upon so many additional factors that their physiological significance declines in proportion to the number of variants acting on an already unphysiological material.

All that can be expected from experiments with muscle mince, therefore, is that they show one particular and well-defined effect under the particular conditions of the experiment, e.g. the catalytic action of fumarate [Stare & Baumann, 1937], or the catalytic action of citrate [Krebs & Johnson, 1937]; and only then can it be assumed with some degree of certainty that such might also be the case in the body.

Certain facts, therefore, have been shown which limit the use of muscle mince for the measurement of the oxidative metabolism. These restrictions however do not apply when washed muscle is used for the study of an isolated enzymic system or when muscle extracts are used for the study of carbohydrate breakdown.

The R.Q. is the outcome of a number of complex reactions. Each of these is, or may be expected to be, differently dependent on the O_2-tension and changes its O_2-dependence differently with time. It is obvious therefore that measurements of the R.Q. should be made within a comparatively short time.

2. EXPERIMENTS IN PRESENCE OF CO_2

All the experiments reported so far have been done with CO_2 absorbed, i.e. with a CO_2-tension of practically zero in the reaction vessel. It has long been recognized that under these conditions results are obtained which in some respects differ fundamentally from those which are gained on the same material when a bicarbonate-containing medium is used buffered by CO_2 in the gas phase [Alt, 1930; Ashford & Holmes, 1931; Dixon, 1934; v. Heyningen, 1935; Laser, 1935]. Lately the role of CO_2 has acquired new importance. Gladstone *et al.* [1935] found that certain aerobic bacteria were unable to grow when CO_2 was rigidly excluded from the medium. Wood & Werkmann [1938; 1940, 1, 2] have shown that propionic acid bacteria utilize CO_2 and have assumed a combination with pyruvic acid, forming oxaloacetic acid and, by reduction, succinic acid. Further data supporting this theory have been added by Krebs & Eggleston [1940] and by Evans & Slotin [1940; 1941]. Krebs & Eggleston found that CO_2-utilization was dependent on the CO_2 pressure, as had also been shown by Elsden [1938] for the formation of succinate by *B. coli*. Another task of CO_2 in tissue metabolism *in vitro* is the neutralization of basic metabolites by the formation of bicarbonate. Elliott & Schroeder [1934], Elliott *et al.* [1935; 1937] and Elliott & Greig [1937] have determined for a variety of tissues and substrates the appearance or disappearance of CO_2 according to the formation of either acid or basic intermediaries. They obtained the value Q_A (μl. CO_2 per mg. per hr.) which is positive when CO_2 is expelled, e.g. by glycolysis of tumours, or negative when bicarbonate is formed as with kidney in presence of sodium succinate or lactate.

In the following section experiments are described in which the influence of CO_2 on the metabolism of tissues is studied. The results are compared with those which were obtained in absence of CO_2.

The following terms are used throughout:

$-Q_{O_2}$ $=\mu$l. O_2 taken up per 1 mg. dry weight of tissue per hour.

Q_S $=\mu$l. CO_2 given out per 1 mg. dry weight of tissue per hour.

Q_A $=\mu$l. CO_2 given out by any acid formation (+) or taken up (−) by any acid disappearance per 1 mg. dry weight of tissue per hour. (In my experiments Q_A was not directly measured by bicarbonate determinations.)

$Q_{CO_2}=Q_S-Q_A=$ respiratory CO_2.

$Q_L^{O_2}$ $=\mu$l. CO_2 given out by lactic acid formation in O_2 per 1 mg. dry weight of tissue per hour.

R.Q. $=Q_{CO_2}/-Q_{O_2}$.

Method

Warburg's differential method [1924; 1925] was used. It was, however, found, if the Q_{O_2} was followed for several hours, that considerable irregularities often occurred in the values obtained. These could be traced to the dilution factor, e.g. to the fact that two lots of tissue slices, suspended in different amounts of fluid which is equimolar with regard to substrate, are exposed to different absolute amounts of substrate unless the ratio of their weights is the same as the ratio of the volumes of fluid in the two vessels. This was, however, difficult to effect and would, furthermore, have increased the experimental error in the vessel containing the small amount of fluid and tissue. Therefore the modified method, as described by Laser & Rothschild [1939], was applied in which the volumes of the pair of vessels differ (about 27 and 11 ml.) while the volumes of fluid (5 ml.) and amounts of tissue slices in each vessel are the same. It is very easy without the use of a torsion balance to select nearly equal portions of tissue for the two different vessels. In this way consistent results were obtained.

The fluid used was Ringer-bicarbonate made up according to Krebs & Henseleit [1932] and the gas 5 % CO_2 in O_2. Temperature 38·5°.

The tissue was mouse kidney and the substrates applied were sodium lactate, succinate, fumarate and pyruvate in final concentrations of $M/50$–$M/100$.

Results

Fig. 10 represents the O_2 uptake of kidney slices to which no substrate was added and shows the Q_{O_2} and the percentage decline of Q_{O_2} over a period of 4 hr. Both curves agree closely with those obtained when CO_2 was absorbed (Fig. 1, curve I; Fig. 3, curve I). The absence of CO_2 can therefore not be held responsible for the decline of O_2 uptake in absence of added substrate and the reasons discussed above for the particular shape of this curve must be considered to hold good also in presence of CO_2.

The shape of the Q_{O_2} curve was, however, considerably altered when substrates were added.

(1) In all the cases examined the initial magnitude of respiration was about the same as the corresponding one determined with absorption of CO_2 in Ringer-phosphate, but it remained much longer at the high initial level or at least decreased much more slowly (Figs. 11–13).

(2) It has previously been observed with all substrates examined (with the exception of succinate) that the rate of oxidation is much lower when the substrate was added after preliminary incubation without substrate than when the substrate was present from the beginning of the experiment. This effect was abolished in all cases of this series,

i.e. the O_2-uptake rose to the initial level of the control when substrate was added to tissues after preliminary incubation of 1 hr. in absence of substrate.

It must be concluded therefore that the relative absence of CO_2 during the preliminary incubation period, as described in Part 1, alters tissues irreversibly within a comparatively short time in such a way that at a later stage they are no longer able to oxidize different substrates at the same rate as they would have done, had they not been deprived of CO_2 during the preliminary period.

It has been seen that in presence of CO_2 the rate of O_2-uptake remains comparatively steady for some time. It remains now to be ascertained whether the O_2-uptake generally can be taken as an indicator for the 'physiological' behaviour of tissue slices during a certain period *in vitro* and whether a relative constancy of Q_{O_2} justifies the assumption that other reactions (only the end-products of which may have been determined) have occurred with a similarly constant rate over the same period of time.

If this were the case, then, the ratio $Q_S : Q_{O_2}$, and the R.Q., should remain constant at least as long as the Q_{O_2} remains constant.

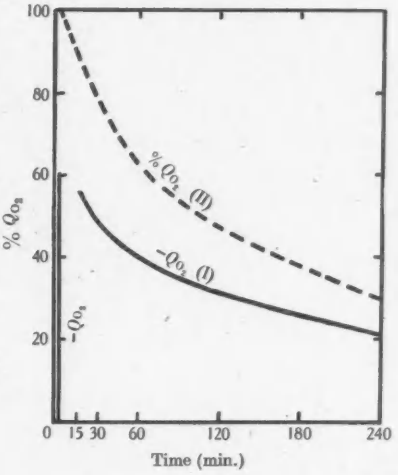

Fig. 10. Mouse kidney slices. $-Q_{O_2}$ (I) and percentage decline of Q_{O_2} with time (II) without addition of substrate.

Although the manometric method employed here can be adapted for the determination of R.Q. and Q_A [Laser & Rothschild, 1939], it has for technical reasons not yet been possible to do the many determinations which

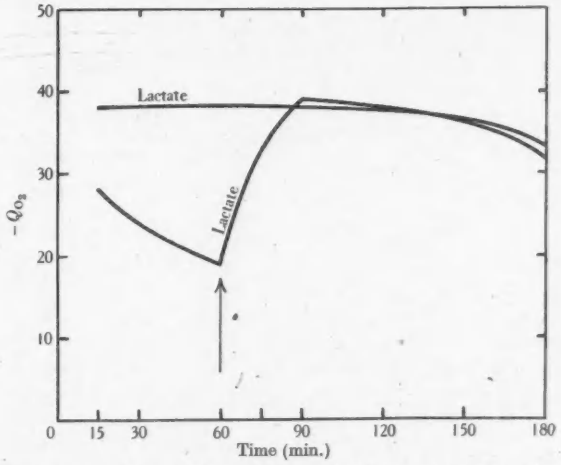

Fig. 11. Mouse kidney slices. $-Q_{O_2}$ on addition of lactate ($M/50$) after preliminary incubation for 60 min. in absence of added substrate and control with lactate present from the beginning.

would have been necessary with either this or one of the other manometric methods for the determination of the R.Q. in presence of CO_2.

The available data, however, for Q_{O_2} and Q_S allow some conclusions to be drawn as to the most likely variations of R.Q. and Q_A, if not as to their absolute values. They

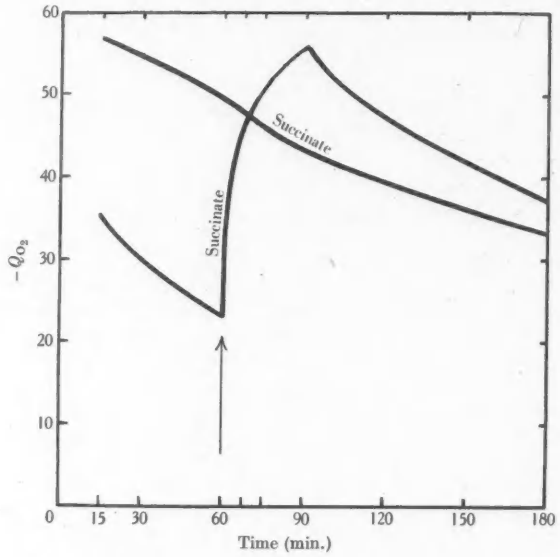

Fig. 12. Mouse kidney slices. Same as Fig. 11 with succinate ($M/50$) as substrate.

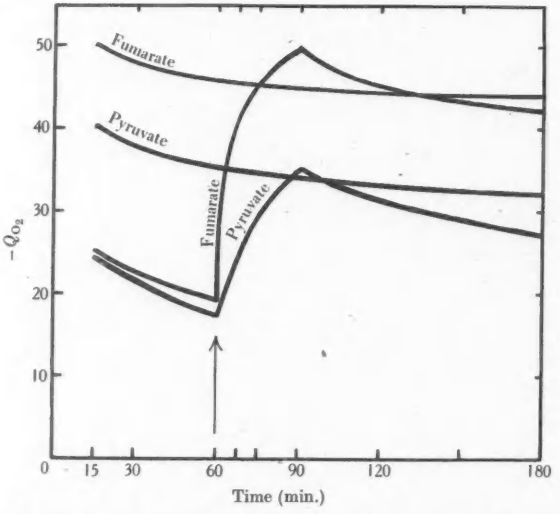

Fig. 13. Mouse kidney slices. Same as Fig. 11 with pyruvate and fumarate ($M/50$) as substrates.

indicate clearly that, in the case of succinate as substrate, of the two values, R.Q. and Q_A, at least one changes considerably with time.

In Fig. 14 Q_{O_2} and Q_S are plotted from the data actually obtained; and Q_A as calculated on the assumption of a steady R.Q. of 0·77 [Elliott *et al.* 1935]. The curves for Q_A and Q_{O_2} (Fig. 14) seem to show some similarity. A considerable divergence, however, becomes clear if they are plotted in a different way (Fig. 15), as percentages of their initial values obtained by extrapolation from the first two readings. The curves then demonstrate that the Q_{O_2} decreased by 52 % in 4 hr. while the Q_A decreased by 91 %. This means that at the end of the experiment while the tissue was still respiring with half its initial rate it had either practically completely lost its power to bind CO_2, for whatever purpose it utilized it at the beginning of the experiment, or the tissue had changed its metabolism in such a way that the participation of CO_2 was longer required.

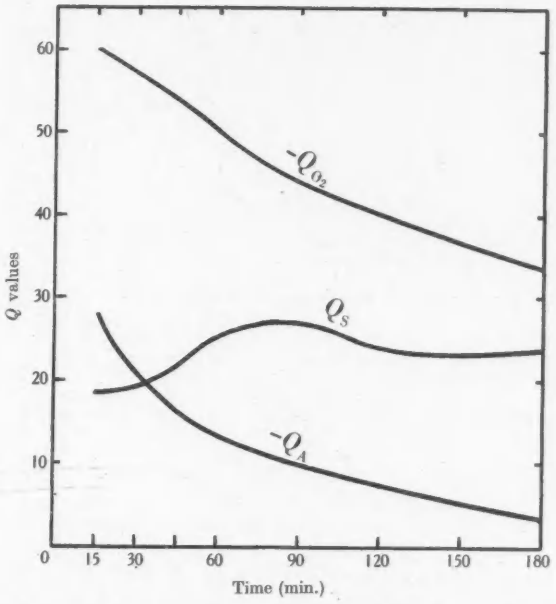

Fig. 14. Mouse kidney slices. Substrate: sodium succinate ($M/50$). $-Q_{O_2}$, Q_S and Q_A. (Q_A, calculated under the assumption of constant R.Q. = 0·77.)

This explanation, however, holds good only if the R.Q. is constant during the whole experimental period, for which there is no proof. The other alternative had therefore to be considered, namely, that Q_A remained constant, in which case $Q_S - Q_A = Q_{CO_2}$. In this way the potential R.Q. can be calculated for different periods. Fig. 16 shows one such hypothetical case where a constant value of $Q_A = -13$ was assumed (the value obtained for Q_A in Fig. 14 at $t = 60$ min.). The curve representing the R.Q. becomes then S-shaped, indicating a change of R.Q. with time. It is irrelevant with regard to the shape of the curve whether this or any other value of those shown for Q_A in Fig. 14 is substituted. The curve for R.Q. will always remain similarly S-shaped provided that Q_A remains constant; thus indicating a change of R.Q. with time: only the position of this curve will be either raised or lowered.

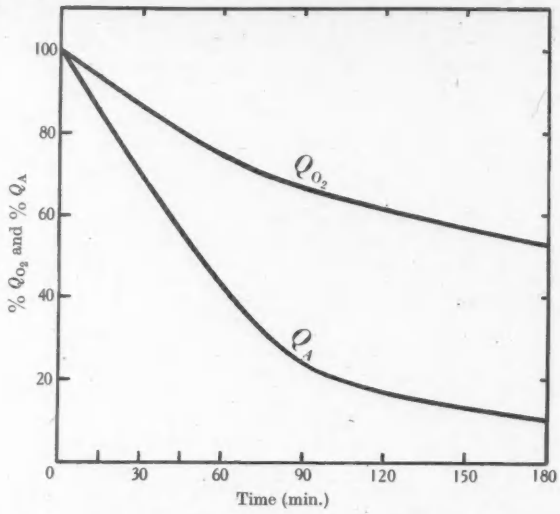

Fig. 15. Mouse kidney slices. Substrate: sodium succinate. Percentage decline of Q_{O_2} and Q_A with time. Q_A calculated as in Fig. 14.

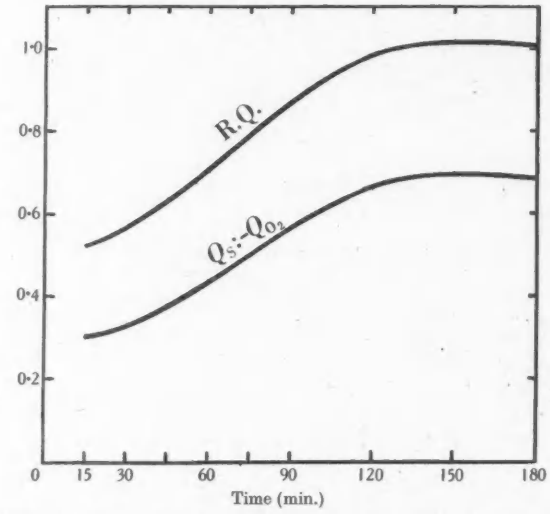

Fig. 16. Mouse kidney slices. Substrate: sodium succinate ($M/50$). $Q_S/-Q_{O_2}$ and R.Q. (R.Q. calculated under the assumption of constant $Q_A = -13$.)

Discussion

The main results of Part 2 of this work, concerning the oxidation of different substrates by kidney slices in presence of CO_2, are: (1) the initial rate of oxidation is much longer maintained or the falling off of its rate is much slower than in absence of CO_2; (2) if substrates are added after a preliminary incubation period of 1 hr. they are oxidized at

the same rate as if they had been present from the beginning. This is not the case if CO_2 is absent during the preliminary incubation period and denotes that a certain CO_2-tension is necessary for the reactivity of tissues to substrates.

It has, furthermore, been proved from the available data that, with succinate as substrate, of the two values, R.Q. and Q_A, at least one undergoes considerable changes during the course of the experiment. A steady R.Q. would result in comparatively great changes in Q_A, during the experimental period, i.e. in the power of the tissue to utilize CO_2. On the other hand, a comparatively steady Q_A would denote a change of R.Q. with time. In fact, it is most likely that the latter is responsible, for the following reasons: (1) added substrate may be oxidized to different end-products as time goes on, (2) the number of other substrates present in the tissues and the rate with which they are oxidized may also vary considerably with time.

There are no data available on the minimum CO_2 tension which would be required for the various processes described by Wood & Werkman, Elsden, Krebs & Eggleston, and Slotin & Evans. It can be assumed, however, that the required tension is comparatively low since in the experiment of Elsden [1938] the formation of succinic acid was only 50 % greater in 100 % CO_2 than in 5 % CO_2/N_2, and complete removal of CO_2 decreased the formation of succinic acid by only 13–40 % according to the different substrates. Similarly, in the experiments of Krebs & Eggleston [1940] the utilization of pyruvate was increased by only 37–94 % if 5 % CO_2 in O_2 was used instead of pure O_2. This indicates that the affinity for CO_2 of the systems in question is very high and that they are able to utilize rapidly CO_2 produced either aerobically by oxidation or anaerobically by fermentation, before it can be effectively removed.

It must be assumed, therefore, that in manometric experiments in which the CO_2 is absorbed, some CO_2 is available to the cells before it can be removed by the absorbent (e.g. KOH). This tension must, however, lie below the minimum required.

With regard to the part played by CO_2 in neutralizing basic metabolites *in vitro* by formation of bicarbonate, comparatively large amounts are required, with some substrates up to 50 % of the respiratory CO_2, and it seems certain that this amount is not readily available under conditions of CO_2-absorption. Even if the use of a phosphate-buffered Ringer solution prevented the medium from becoming alkaline, the *p*H inside the cells might under these conditions be considerably shifted to the alkaline side in the absence of glycolysis.

Since differences corresponding to the presence or absence of CO_2 exist in the metabolic rate of tissue slices, and since qualitative differences may occur depending on very slight differences of CO_2 tension, especially if this is very small, the determination of the R.Q. in tissue slices by the method of Warburg & Yabusoe [1924] may produce misleading results because O_2-uptake is measured in one vessel with KOH and this value is correlated with another obtained in absence of KOH.

The question of using serum instead of Ringer-bicarbonate has not been dealt with in this paper. It is known that some differences of metabolism occur according to which of the two media is used. I found that aerobic glycolysis ($Q_L^{O_2}$) of rat retina is 45 in Ringer-bicarbonate and only 25 in serum. Although it is tempting to assume that serum is a more physiological medium than Ringer-bicarbonate solution, serum differs in many respects from plasma. It is conceivable, therefore, that some of the physiological advantages of serum as compared with Ringer might be offset by some intrinsic effect of serum.

SUMMARY

O_2-uptake and CO_2-production by kidney slices in absence (Part 1) and in presence (Part 2) of CO_2 have been examined for different periods of prolonged experiments (4 hr.).

(1) In absence of CO_2:

(*a*) The shape of the Q_{O_2} curve in absence of added substrate shows a characteristic decline with time. The reasons for this particular shape of curve are discussed.

(*b*) The rate of O_2-uptake without added substrate declines with time more rapidly in 100 % O_2 than at lower O_2-tensions; thus the rate of respiration after 60 min. is higher at O_2-tensions between 10 and 20 % O_2 than in 100 % O_2.

(*c*) The rate of O_2-uptake in presence of added substrate expressed as function of the O_2-tension changes with time in a particular way for each substrate.

(*d*) The rate of O_2-uptake when substrates are added after preliminary incubation for 1 hr. in absence of added substrate is lower (except in the case of succinate) than the Q_O of the control in presence of substrate at the beginning of the experiment and, in some cases, lower than the Q_{O_2} of the control at the time of adding the substrate.

(2) In presence of CO_2:

(*a*) The shape of the Q_{O_2} curve without added substrate is the same as in absence of CO_2.

(*b*) The rate of O_2-uptake in presence of substrates is either constant for several hours or falls off much less than in absence of CO_2.

(*c*) The rate of O_2-uptake when substrates are added after preliminary incubation without substrate for 1 hr. becomes in all cases practically the same as the Q_{O_2} of the corresponding control at the beginning of the experiment.

(*d*) Analysis of the data obtained for Q_{O_2} and Q_S reveals that of the two values, R.Q. and Q_A, at least one undergoes considerable changes with time.

The main conclusions, therefore are:

I. Maximum activity of tissue slices and reactivity to substrates in absence of CO_2 are maintained only for a comparatively short time.

II. The presence of CO_2 stabilizes the rate of O_2-uptake of tissue slices in presence of substrate for several hours and safeguards their ability to oxidize substrates which are added after incubation in absence of substrate.

III. A comparatively steady Q_{O_2} does not indicate a similar steadiness of other reactions (only the end-products or final figures of which have been determined) occurring during the experimental period.

IV. The results summarized under (1) and (2) make it obvious that the R.Q. can only be determined with any expectation of physiological significance in experiments of short duration (e.g. 15–30 min.) and under conditions of equal CO_2-tension in all the vessels necessary for 1 determination.

V. Significant quantitative and qualitative discrepancies exist between the oxidative powers of untreated tissue mince (or pulp) and tissue slices which limit the use of mince as a physiological material in the study of the oxidative metabolism of tissues.

VI. The variations of the catalytic activity of different oxidative systems with time and with alterations in O_2- and CO_2-tension, the decreasing ability of tissue slices with time to utilize CO_2 and the—mostly uncontrollable—variations in interaction of different enzymic systems with time, which result in changes of the R.Q., ought to be considered in each particular case when assessing the degree of physiological significance which can be attached to experiments with tissue slices *in vitro*.

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37. Vitamin C in Rose Hips

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(*Received 19 January 1942*)

The very high concentration of vitamin C present in rose hips has been observed by a number of Russian workers [see Iwanoff & Bukin, 1937]. 1380 mg. of ascorbic acid per 100 g. were found in *R. acicularis* and 1540–2580 mg./100 g. in *R. dahurica*. In a more detailed abstract in the same publication, Bukin and Zubkova record the interesting observation that rose hips found in northern latitudes contain more vitamin C than those of species growing further south. The richest samples of *R. cinnamomea* and *R. acicularis*, growing in the northern and middle parts of the U.S.S.R., contained 4750 mg. of vitamin C/100 g. of raw fruit pulp, equivalent to 14·6 % of the dried pulp, while *R. canina*, propagated chiefly in the south, contained at best the equivalent of 2·2 % vitamin C in the dried pulp. However, not only were the species growing in the north richer in vitamin C than those in the south, but specimens of the same species contained more vitamin C when grown in a more northerly latitude. For example, *R. rubiginosa* from Minsk was found to contain 3880 mg./100 g. compared with samples containing 1400 mg./100 g. from Maikop, further to the south. Another observation seemed to indicate that rose hips growing at high altitudes might be richer in vitamin C than the same species lower down. *R. canina* in the Caucasus mountains was found with 1480 mg./100 g., while hips of the same species taken from the Black Sea shore contained only 470 mg./100 g.

During the past autumn, specimens of a large number of the rose species which grow in this country have been tested for their content of vitamin C. As far as possible only ripe fruit was collected; it was sent to the laboratory by post and there placed in cool storage. In most cases the hips were analysed within a few days of picking. The range of results given below would seem to explain many of the anomalous figures which may arise from individual analyses and also most strikingly confirm the Russian conclusions that species with a more northerly habitat contain higher concentrations than those which grow further south.

Experimental

(1) *Method of analysis*

The 'seeds' or pips—strictly, achenes—of rose hips are hard and difficult to grind and the amount of vitamin C in them is small. The hips were therefore split open, the seeds removed and the proportion of seeds to total fruit determined. Since the number of fully developed seeds fluctuated even in the hips of one species and the hips of some species are drier and less fleshy than others, the proportion of seeds may vary within wide limits. In the present work, the percentage of seeds has been recorded and the analysis of vitamin C carried out on the flesh only. The date of analysis seldom differed by more than a few days from the date of picking; when delay was unavoidable, the hips were kept in cool storage.

To analyse the flesh, a 5 g. sample was ground to an impalpable consistency in a mortar with a small amount of powdered quartz and in the presence of a 5 % solution of H_2SO_4 containing 2 % freshly dissolved metaphosphoric acid. It is important that the hip flesh be covered with the sulphuric-metaphosphoric acid solution as quickly as possible since enzymes start to destroy the ascorbic acid as soon as the fruit is broken up. The volume of suspension was made up to 500 ml., of which the acid solution comprised about 100 ml.,

the remainder being water. Vitamin C was then determined by titrating 5 ml. of a freshly prepared solution of 2:6-dichlorophenolindophenol with the clear centrifuged solution. The dye was standardized so that 5 ml. were equivalent to about 1 mg. of ascorbic acid.

In most cases the hip solution was only slightly coloured and the end-point was good. Hips from *R. spinosissima* and its allies, however, gave red pigmented solutions which were titrated in chloroform according to the method of McHenry & Graham [1935]. All the figures shown in this paper represent only reduced ascorbic acid. On those occasions when treatment with H_2S was carried out, no increase in vitamin C could be detected. It appears, therefore, as is the case with acid fruits in general, that most of the vitamin occurs in the reduced form.

(2) *Vitamin C in British rose species*

If rose hips are divided into four groups according to the average ascorbic acid content of the flesh of the ripe fruits, an interesting division of species occurs. In Table 1 below,

Table 1. *Range of vitamin C content of the flesh of ripe hips of British roses*

Species	No. of samples	Ascorbic acid, mg./100 g. flesh		% pips	
		Range	Average	Range	Average
Group (*a*), average values more than 1000 mg./100 g.					
Rosa Afzeliana	19	500–1600	1000	21–46	36
R. coriifolia	15	750–1820	1080	24–51	35
R. mollis	11	950–1560	1260	24–58	37
R. Sherardi	19	520–1870	1240	24–55	34
Group (*b*), average values between 500 and 1000 mg./100 g.					
R. canina	68	200–1010	550	25–52	38
R. dumetorum	38	240–990	590	28–55	38
R. rubiginosa	6	550–1230	810	35–49	41
R. tomentosa	16	340–1180	690	27–55	36
Group (*c*), average values between 250 and 500 mg./100 g.					
R. agrestis	2	420–500	460	33–37	35
R. micrantha	7	200–650	400	24–49	42
R. obtusifolia	9	150–600	420	33–50	42
R. spinosissima	7	150–760	340	21–29	24
Group (*d*), average values under 250 mg./100 g.					
R. arvensis	25	10–170	80	26–53	37
R. stylosa	9	70–380	190	33–48	40

an arbitrary division of average values into (*a*) those over 1000 mg./100 g., (*b*) those between 500 and 1000, (*c*) those between 250 and 500, and (*d*) those under 250 mg. of vitamin C/100 g. was made. After grouping the species in this way, however, it clearly appeared that roses indigenous to Scotland and the north of England fell in the highest group, which included 64 samples. Of these, only 15 were obtained south of the Lake District, 9 being in North Wales and 4 cultivated. The second group contained species with a characteristically English distribution, for the most part extending only to the south of Scotland and there becoming rare. Only 3 of the 128 samples in this group were picked in Scotland. The third group is heterogeneous; *R. agrestis* occurs mainly in southern England, *R. micrantha* grows in England and becomes rare and local in Scotland, *R. obtusifolia* extends as far north as Yorkshire, while *R. spinosissima* has a general distribution but is mainly coastal. Omitting *R. spinosissima*, none of the samples of the other species in group (*c*) came from further north than mid-Wales. The fourth group comprised roses almost confined to southern England the most northerly samples coming from North Wales.

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(3) *Vitamin C in foreign rose species*

In addition to the results for rose species indigenous to this country shown in Table 1 hips of a number of foreign roses have been analysed. These were all from bushes cultivated in Surrey and in few cases was more than a single sample examined.

The foreign rose species also fall into 4 groups which do not correspond exactly with those into which the British roses can be divided. These have average vitamin C content in mg. per 100 g. of flesh as follows: group (1) over 1500; group (2) 1500–1000; group (3) 1000–500; group (4) below 500. In the following list the figure for the vitamin C content is followed in brackets by the percentage of pips. Group (1), *R. elymaitica* 2080 (20), *R. Fedtschenkoana* 4800 (45), 3050 (35); *R. macrophylla* 1990 (23); *R. Moyesii* 1600 (22), 1250 (26); *R. nutkana* 1660 (33). Group (2), *R. caudata* 1120 (17); *R. cinnamomea* 1180 (23), 1070 (33); *R. manca* 1100 (20); *R. pisocarpa* 1100 (32); *R. pomifera* 1020 (30); *R. rubrifolia* 1030 (37); *R. Sweginzowii* 1430 (24); *R. therebinthinacea* 1370 (28). Group (3) *R. acicularis* var. *Engelmannii* 910 (33); *R. altaica* 520 (20); *R. baicalensis* 590 (30); *R. calocarpa* 930 (29); *R. rugosa* 970 (25), 540 (27); *R. salaevensis* 920 (31); *R. virginiana* 880 (33). Group (4), *R. Helenae* 260 (31).

The first group of foreign roses includes hips very much richer in vitamin C than any of the native species. Of the native material, a specimen of *R. Sherardi* var. *suberecta* contained 1870 mg./100 g., but another sample, possibly of this variety collected late in the year and incapable of exact identification in the absence of leaves, contained 2810 mg. 100 g. This was the richest native sample, but it was far surpassed by a Turkestan species *R. Fedtschenkoana*, grown at Kew, the fresh flesh of which contained 4800 mg. of ascorbic acid per 100 g., i.e. 4·8 % of vitamin. *R. Fedtschenkoana* was the only species that gave a figure comparable with that obtained in Russia by Bukin and Zubkova for *R. cinnamomea*. In England the latter species was not outstanding and the reasons for its much lower vitamin content, whether varietal differences or climatic effects, remain obscure. The only foreign rose with a vitamin C content of less than 500 mg. per 100 g. of flesh was *R. Helenae*, the only foreign species tested that belongs to the section *Synstylae*, which includes our native *R. arvensis*. It is notable that many of the foreign rose hips contained a lower percentage of pips than most of the British species. Incomplete fertilization may account for part of the difference, but often it is associated with an elongated shape of the hips as in *R. Moyesii*, *R. Sweginzowii* and *R. caudata* or with thick flesh as in *R. rugosa*.

Discussion

It appears quite clearly from Table 1 that a close correlation exists between the latitudinal range of British rose species and the vitamin C content of their hips. All those with a high vitamin content, group (*a*), extend throughout Scotland and into north England; group (*b*) is intermediate and includes species common in England but thinning out in southern Scotland; group (*d*) is practically confined to southern England. With the exception of *R. spinosissima* which is generally distributed around our coasts, group (*c*) falls into line in so far as the remaining species have a predominantly southern distribution.

The reasons for this latitudinal variation are not yet clear. Rainfall, sunshine and length of day may play their part, and possibly soil and other environmental factors contribute to the wide range of vitamin content observed in some species. Nevertheless ascorbic acid content appears to be a constitutional character not readily altered by a change of environment. Thus, transplantation of a bush of *R. mollis* var. *typica* from Westmorland to a garden in Middlesex does not appear to have affected the vitamin content of the hips. It is likely also that much of the variability of certain species is constitutional and not induced by environmental factors as the genetical relationships within this polymorphic genus are known to be complex.

Part of the variation may be attributed to varying degrees of ripeness in the hips. Quiller [1939] reported that the ascorbic acid content of rose hips increases during maturation and decreases when the fruits are over-ripe. In our studies the changes in vitamin C content have not been followed from the green stage to full ripeness, but an increase was found from the orange ripe stage to full ripeness in the hips of a bush of *R. rubiginosa*. The sampling dates and vitamin C contents were as follows: 12. ix. 41, 635; 14. x. 41, 745; 12. xi. 41, 995 mg./100 g. of flesh. A bush of *R. dumetorum*, first sampled when the hips were red ripe, gave the following results: 29. viii. 41, 920; 26. ix. 41, 850; 20. x. 41, 990; 14. xi. 41, 930 mg./100 g. of flesh. No marked decline in the vitamin content of the over-ripe hips of the final sample was evident; had there been much loss of vitamin, it was masked to a large extent by the drying and shrivelling of the fruit. Variation due to the stage of ripeness was kept to the minimum by asking those collecting specimens to supply ripe hips and by a judicious selection of 'ripe' hips from each sample.

Another possible source of variation is the difficulty of distinguishing hybrids from their parent species, especially when only scanty material is available. It is probable that more hybrids exist than have so far been recognized, but this and other taxonomic questions will be discussed elsewhere. The nomenclature adopted is that of Wolley-Dod [1930–31].

Summary

1. A survey has been made of the vitamin C content of the ripe hips of a number of British roses. Some foreign roses grown in this country have also been investigated.

2. A number of common species contain large amounts of vitamin C, often exceeding 1 %, in the flesh of the ripe hips.

3. The species indigenous to Scotland and northern England appear to contain higher concentrations of vitamin C than those roses commonly found in the more southern parts of this island.

The authors must acknowledge their gratitude to those members of the British Ecological Society who co-operated so enthusiastically in the collection of material, and to Mr G. Blackman, Secretary of the War Biology Committee and others who gave assistance. They wish particularly to express their appreciation of the help given by the late Sir Arthur Hill, Director of the Royal Botanic Gardens, Kew, and to Dr Tincker of the Royal Horticultural Society's gardens at Wisley. They would also like to take the opportunity of acknowledging the expert technical assistance of Mr J. Croucher without which the completion of the analytical work would not have been possible.

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38. The Preparation and Properties of a Specific Polysaccharide from *Bact. typhosum* Ty_2

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With an Addendum by **J. St L. Philpot**, *From the Department of Biochemistry, Oxford*

(*Received 12 March 1942*)

The observation of Dochez & Avery [1917] that culture filtrates from the three main types of *Pneumococcus* contain a soluble substance which gives specific precipitation with the homologous immune serum has led to studies on many types of *Pneumococcus* which have established the importance of polysaccharides in determining the type specificity of certain bacterial antigens [Heidelberger & Avery, 1923; Heidelberger *et al.* 1925; Avery & Goebel, 1933]. Artificial carbohydrate-protein antigens have been synthesized by Goebel [1939; 1940], in which the specificity of the antigenic complex may be determined not only by a complex polysaccharide but by a relatively simple specific pattern unit such as an aldobionic acid. This observation has stimulated inquiry into the nature of the component carbohydrate residues and structure of the polysaccharide haptens of bacterial antigens.

Morgan [1936; Morgan & Partridge, 1940; 1941] has shown that the dominant smooth antigen of *B. dysenteriae* (Shiga) consists of a complex of a polysaccharide, a phospholipin and a conjugated protein, in which the polysaccharide, which appears to determine the specificity of the complete antigen, is composed of *d*-galactose, *l*-rhamnose and *N*-acetylglucosamine units [Morgan, 1938].

The antigens of *Bact. typhosum* and the closely related *Bact. typhi-murium* were first isolated and studied by Boivin *et al.* [1933], Raistrick & Topley [1934] and Topley *et al.* [1937] and appeared to consist predominantly of a polysaccharide linked to an insoluble nitrogen- and phosphorus-containing substance believed to be a phosphatide. Freeman *et al.* [1940] obtained the antigens of these organisms in a state approaching chemical homogeneity by modifications of the methods of Boivin and Raistrick & Topley, and in the case of *Bact. typhosum* the antigen was shown to exist as a complex of a polysaccharide, a polypeptide and a small amount of a lipid component [Freeeman & Anderson, 1941].

In the present paper the preparation in a pure state, and properties of the polysaccharide component of *Bact. typhosum* Ty_2 are described in detail. Degradation of the polysaccharide and identification of its component hexose residues have resulted in characterization of about 78% of the component carbohydrate residues of the complex substance. In view of the observation of Avery & Goebel [1933] that the specific polysaccharide of *Pneumococcus* Type I exists in the bacterial cell as an acetyl polysaccharide which may be isolated as such and has the power, not possessed by the deacetylated compound, of stimulating the production of specific antibodies on injection into mice, the influence of acetyl groups introduced into the polysaccharide molecule on its precipitability by *Bact. typhosum* O-antiserum has been studied.

Experimental

The dried bacterial cells of *Bact. typhosum* Ty_2 used in this investigation were grown on a synthetic medium as described by Freeman *et al.* [1940].

Preparation of the specific polysaccharide

The specific polysaccharide of *Bact. typhosum* Ty_2 may be prepared either from the complex bacterial antigen, isolated from the bacterial cells by tryptic digestion [Freeman *et al.* 1940], by means of hydrolysis with dilute acetic acid, or directly from the dried bacterial cells by extraction with dilute acetic acid. The bulk of the crude polysaccharide used in these experiments was obtained by hydrolysis of the tryptic digest antigen in the course of investigation of the nature of the latter [Freeman & Anderson, 1941].

(*a*) *Preparation from tryptic digest antigen.* The tryptic digest antigen, usually in batches of about 5 g., was dispersed by grinding in a mortar with sufficient 0·1 *N* acetic acid to give a 1 % solution which was shaken mechanically until a homogeneous opalescent solution was obtained. This was heated on the boiling water bath, under reflux for 12 hr.; during the hydrolysis the solution first clarified a little (30 min.), but after 2 hr. it became very opalescent and the insoluble polypeptide component of the antigen began to flocculate out. After about 8 hr. heating the flocculation appeared to be complete and there was no further deposition of precipitate and no further change of optical rotation during the last 2 hr. of hydrolysis. The insoluble polypeptide fraction was removed by centrifuging in an angle centrifuge for about 4 hr. at 2000–3000 r.p.m. giving a slightly opalescent brownish coloured supernatant solution, which was concentrated *in vacuo* at low temperature to one-third of the original volume. Recentrifuging at this stage gave a further small precipitate of insoluble polypeptide. The crude polysaccharide was precipitated from the supernatant solution by addition of absolute alcohol to 86 % by weight. Addition of alcohol to 48 % gave slight precipitation, alcohol to 60 % produced a bulky flocculent precipitate and after increasing the alcoholic content to 86 % by weight flocculation of the crude polysaccharide appeared to be complete. The crude polysaccharide ('Polysaccharide F 86 %') was centrifuged off, washed twice with absolute alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The crude polysaccharide was a water-soluble, almost colourless amorphous substance which corresponded to about 57 % of the antigenic complex. It contained 3·6 % N, 1·6 % organic P, and gave 55 % of reducing sugars on hydrolysis with 2 *N* HCl for 2 hr. at 100° in a sealed tube [Hagedorn & Jensen, 1923], non-volatile ash = 20·2 %. On electrodialysis for 48 hr. against distilled water through cellophane membranes a sample of the crude polysaccharide yielded 39 % of alcohol-precipitated polysaccharide F 86 % containing 1·0 % N and 3·2 % non-volatile ash. In a further experiment after 120 hr. electrodialysis 33 % of a partially purified polysaccharide F 86 %, containing 0·5 % N and 2·6 % non-volatile ash, was obtained.

Alcoholic fractionation of polysaccharide. A mixture of 0·196 g. of two samples of electrodialysed polysaccharide was dissolved in 10 ml. water ($[\alpha]_{5461}^{17^\circ} = +134^\circ$, uncorrected for ash) and fractionally precipitated with absolute alcohol. Addition of alcohol to 45 % by weight produced a small amount of precipitate which was centrifuged off and the alcoholic content of the clear supernatant solution increased to 54 %. The process was repeated until fractions corresponding to alcoholic concentrations of 0–45, 45–54, 54–60, 60–70, 70–80 and 80–99 % were obtained, these were washed twice with alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide. The yields and properties of the fractions obtained are summarized in Table 1.

N, P, ash, reducing sugar value and specific rotation determinations, were carried out as described by Freeman & Anderson [1941], and other analyses were by Dr G. Weiler. Except where otherwise stated analytical results are quoted on an ash-free basis. Melting-points are uncorrected.

The analytical data of the fractions isolated by alcoholic fractionation of the electrodialysed polysaccharide afford good evidence that the latter consists predominantly of a single polysaccharide. The mother liquor fraction F 80–99 % contains the bulk of the

Table 1. *Alcoholic fractionation of electrodialysed polysaccharide*

Polysaccharide

0·196 g. 0·072 g.: F 86 % (*a*) N = 1·0 %, Ash = 3·2 %
0·124 g.: F 86 % (*b*) N = 0·5 %, Ash = 2·6 %

Dissolved in 10 ml. water, solution centrifuged to remove a trace of residue

$[\alpha]_{5461}^{17°} = +134°$

Alcohol added to:

	45 %	54 %	60 %	70 %	86 %	99 %
	F 0–45 %	F 45–54 %	F 54–60 %	F 60–70 %	F 70–80 %	F 80–99 %
	0·0035 g.	0·0012 g.	0·0342 g.	0·075 g.	0·032 g.	0·0254 g.
Yield (%)	1·8	0·6	17·5	38·2	16·1	13·0
Non-volatile ash %	—	—	1·0	0·8	2·0	11·6
N (%)	—	—	0·2	0·2	0·6	1·5
R.V. (%)	—	—	78	77	76	77
$[\alpha]_{5461}^{17°}$	—	—	+149°	+150°	+152°	+166°

Recovery = 87·2 %.

ash and has a higher nitrogen content than fractions F 54–60 %, F 60–70 % and F 70–80 % which are practically identical and indicate that the purified polysaccharide contains 0·3 % N, has a reducing value (on hydrolysis with 2*N* HCl for 2 hr. at 100° in a sealed tube) of 77 %, specific rotation $[\alpha]_{5461}^{17°}$ = about +150°, non-volatile ash = 1–2 %. (The specific optical rotations quoted in Table 1 are based on microdeterminations in a 0·5 dm. tube with readings of 0·3–0·4° and are subject to an error of ±5 %.) The low and variable nitrogen contents of the fractions isolated by alcoholic fractionation as compared with the constant reducing values suggest that the nitrogen is present as an impurity and does not form an essential molecular constituent, but nitrogen to the extent of 0·2–0·4 % has persisted after fractionation with other solvents, e.g. acetic acid and even after acetylation of the polysaccharide and subsequent reconstitution from the acetylated derivative in a virtually unchanged form. In order to eliminate impurities and inorganic salts from the crude polysaccharide repeated alcoholic fractionation between the limits of alcoholic concentration of 45–50 and 86 % have been found to be effective as the results of Table 1 clearly show.

Acetic acid fractionation of polysaccharide. 1·15 g. of crude polysaccharide which had previously been twice fractionated between the limits of 42 and 86 % of alcoholic concentration (N = 2·2 %, R.V. = 68 %, non-volatile ash = 6·9 %) were dissolved in 100 ml. water and heated on the boiling water bath with a little animal charcoal for about 30 min. to decolorize. The solution was freed from charcoal by filtration and concentrated *in vacuo* to 12 ml. It was then fractionated with alcohol between the limits of 45 and 86 % concentration of alcohol by weight, i.e. alcohol was added to 45 % and any precipitate produced centrifuged off and rejected, the alcoholic concentration was then increased to 86 % and the bulk of the polysaccharide thereby thrown out of solution; this was centrifuged off and redissolved in 12 ml. water and the procedure repeated three times in all. When most of the inorganic salts had been eliminated from the polysaccharide by alcoholic fractionation, it was necessary to assist flocculation by addition of a few drops of glacial acetic acid. At this stage the fractionated polysaccharide gave no precipitate with any of the following reagents: neutral lead acetate, basic lead acetate, 2 % phosphotungstic acid in 5 % H_2SO_4, and 5 % mercuric acetate solution. The polysaccharide was then fractionated with glacial acetic acid; to the solution, 11·3 g. in weight, 60 ml. glacial acetic acid were added and gave no precipitate or opalescence. Addition of 70 ml. glacial acetic acid, i.e. 86·7 % by weight, gave an opalescence which flocculated and deposited a precipitate. This was centrifuged off and the acetic acid concentration of the supernatant

solution then increased to 91·8 %. This procedure was continued until 94 %, 96·2 % and finally an acetic acid mother liquor fraction F 96–99 % were obtained. The fractionation and analytical data of the fractions are summarized in Table 2.

Table 2. *Alcoholic and acetic acid fractionation of crude polysaccharide*

Polysaccharide
1·1g. F 42–86 %/42–86 %
N = 6·9 %, R.V. = 68 %, Ash = 6·9 %
Three alcoholic fractionations between 45 % and 86 % alcoholic concentration by weight

F 42–86 % (2)/45–86 % (3)
Redissolved in water and treated with glacial acetic acid

Alcoholic M.L. residue
F 86–99 %
0·155 g. (14·1 %)
N = 5·3 %. Ash = 5·2 %

Acetic acid conc.	86·7 %	91·8 %	94·0 %	96·2 %	99 %
	F 0–86·7 %	F 86·7–91·8 %	F 91·8–94 %	F 94–96·2 %	F 96·2–99 %
	0·138 g.	0·344 g.	0·082 g.	0·049 g.	0·066 g.
Yield (%)	12·5	31·3	7·5	4·5	6·0
N (%)	0·2	0·3	0·3	0·6	1·4
Non-volatile ash (%)	1·4	1·1	2·9	2·1	6·6
$[\alpha]^{19°}_{5461}$	+145	+140	+142	+123	—

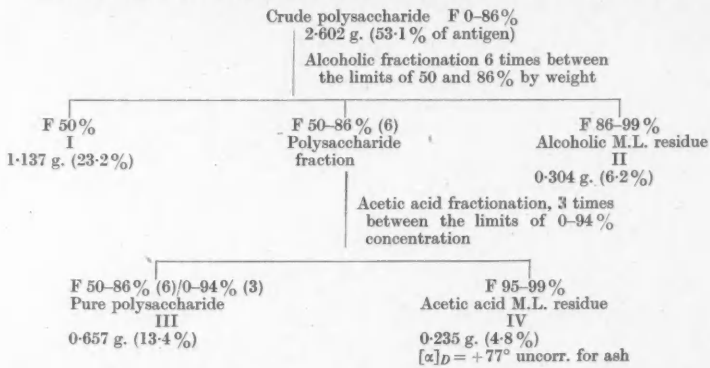
The results of the acetic acid fractionation confirm those of the previous alcoholic fractionation. There is again no evidence of the presence of more than one polysaccharide. The fractions F 0–86·7 %, F 86·7–91·8 %, F 91·8–94 % are virtually identical and agree with the corresponding alcoholic fractions in containing 0·2–0·3 % N and 1–2 % non-volatile ash. As in the case of the alcoholic fractionation the mother liquor fractions F 94–96·2 % and F 96–99 % contain the bulk of the inorganic impurities as well as much more nitrogen than the main fraction F 0–94 % which accounts for the bulk of the polysaccharide.

From the results of the alcoholic and acetic acid fractionations of the crude polysaccharide it was concluded that a satisfactory means of purification of the latter was afforded by repeated alcoholic fractionation (6 times) between the limits of 50–86 % alcohol by weight, followed by acetic acid fractionation (3 times) between the limits of 0–94 % acetic acid. The purified polysaccharide was then twice reprecipitated from aqueous solution with alcohol to free it from acetic acid and washed twice with absolute alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The scheme of fractionation of the crude polysaccharide is summarized in Table 3; in a typical fractionation 2·602 g. crude polysaccharide obtained by hydrolysis of 5·07 g. tryptic digest antigen gave 0·657 g. pure polysaccharide or 13·4 % of the antigen. Percentage yields are in terms of tryptic digest antigen used as starting material (5·07 g.).

Fractions I–IV (Table 3) were tested by means of the serological precipitation reaction to confirm that the by-product fractions I, II and IV were relatively free from specifically reacting substances as compared with the pure polysaccharide fraction III. A series of dilutions of the substances from 1 in 250 to 1 in 250,000 were mixed with equal quantities (0·15 ml.) of *Bact. typhosum* O-antiserum (for which the author is indebted to Lt.-Col. R. F. Bridges of the Medical Research Council Standards Laboratory, Oxford) and maintained at 37° for 2 hr., room temperature for a further 1 hr. and then observed after standing in a refrigerator for 18–20 hr. Fraction III (Polysaccharide F 50–86 % (6)/0–94 % (3)) precipitated to a dilution of 1 in 125,000, fraction I (F 50 %) gave a slight precipitation to a dilution of 1 in 25,000, whilst fractions II and IV gave no precipitation even at dilutions of 1 in 500. These observations show that the method of fractionation of

Table 3. *Preparation of purified polysaccharide from the crude product*



the crude polysaccharide which has been developed provides a sharp division into non-specific and specific fractions, the bulk of the latter being concentrated in fraction III.

(*b*) *Preparation of specific polysaccharide directly from the dried bacterial cells.* A crude polysaccharide was obtained from the dried bacterial cells of *Bact. typhosum* Ty_2 by means of extraction of the cells with dilute acetic acid at 100° (cf. White [1929] and Morgan [1936]). 15·7 g. of *Bact. typhosum* Ty_2 were dispersed in 200 ml. 0·1 *N* acetic acid and the mixture heated on the boiling water bath for $1\frac{1}{2}$ hr. under a reflux condenser. The suspension was then cooled and centrifuged (2000 r.p.m. for 2 hr.) and the supernatant solution passed through a Chamberland L_2 candle. The unextracted residue from the centrifuge tubes was redispersed in a further quantity of 0·1 *N* acetic acid and the process repeated 4 times in all. Determinations of optical rotation on the extracts after filtration showed that the 3rd and 4th extracts contained little or no polysaccharide. The combined filtrates were concentrated *in vacuo* to about 1/8th vol. and treated with alcohol to 86% by weight to precipitate the crude polysaccharide. The crude product was submitted to alcoholic fractionation in a manner similar to that described above (Table 1) and from the nitrogen contents and reducing values on hydrolysis of the separated fractions it was again apparent that only a single polysaccharide was present which was probably identical with that obtained by hydrolysis of the isolated antigenic complex; the polysaccharide was purified by repeated alcoholic fractionation (6 times) between concentrations of 50–86% followed by 3 acetic acid fractionations between concentrations of 0–94% as described above.

Properties of the polysaccharide

The polysaccharide, prepared as described, was a water-soluble, colourless amorphous substance. It contained N, 0·2–0·4%, and P, 0·4–0·9%, its small reducing value of 1·1%, before hydrolysis, increased to 83% as apparent glucose after hydrolysis with 2 *N* HCl for 2 hr. at 100° in a sealed tube (Hagedorn and Jensen). It was highly dextrorotatory $[\alpha]_D^{16°}$, +114–115°; $[\alpha]_{5461}^{16°}$, +135° (uncorr. for ash). Sulphur was absent and the ash content was *ca.* 2%. On hydrolysis the polysaccharide yielded 3% of volatile fatty acid (calculated as acetic acid) indicating the presence of acetyl residues in the molecule. (Samples of polysaccharide for determination of acetyl were several times reprecipitated from aqueous solution with alcohol and then dried at 1–2 mm. and 100° for 1 hr. to eliminate traces of acetic acid due to fractionation with that solvent.) (Found: C, 44·41; H, 7·12; non-volatile ash, 2·4%, uncorr. for ash. $C_6H_{10}O_5$ requires C, 44·41; H, 6·22%.) The analytical values of the polysaccharide varied within narrow limits from batch to

batch and according to the source of the substance, i.e. whether isolated from the 'tryptic digest antigen' or directly from the dried organisms. The above results represent a typical sample prepared by hydrolysis of the antigenic complex. A typical sample isolated directly from the dried bacterial cells gave on analysis C, 44·48; H, 7·04; N, 0·4; P, 0·9; reducing sugar value (on hydrolysis), 83; acetyl, 6·0; non-volatile ash, 1·8 % (results uncorrected for ash).

The average yield of purified polysaccharide from the antigenic complex was about 13 %, equivalent to 0·6–0·7 % of the dried bacterial cells, although in one experiment in which the antigenic complex was hydrolysed for only 2 hr. with 0·1 *N* acetic acid at 100°, the yield reached 21 % of the antigen. The polysaccharide was obtained directly from the dried bacterial cells in a yield of 0·65–0·75 %. The preparation of the polysaccharide directly from the bacterial cells is obviously much quicker and less expensive than that involving intermediate isolation of the antigenic complex, but much of the material used in this investigation was isolated by hydrolysis of the antigen in the course of a study of its chemical nature [Freeman & Anderson, 1941].

The polysaccharide dissolved readily in water, giving water-clear, colourless solutions; they failed to give positive Millon's or α-nitroso-β-naphthol tests for tyrosine and the biuret test was also negative. Ninhydrin tests and the Sakaguchi reaction for arginine were negative. The polysaccharide gave a very strongly positive Molisch's reaction, but gave no coloration with iodine and failed to reduce Fehling's solution. There was no evolution of furfuraldehyde on heating with 12 % HCl. Bial's reaction and Tollen's phloroglucinol test for pentoses were also negative as were the Seliwanoff test and Foulger's test for ketoses. Tollen's naphthoresorcinol test was negative, indicating the absence of uronic acid residues. 5 mg. polysaccharide gave no appreciable development of colour on heating with skatole and conc. HCl for 30 min. at 38–40°, under which conditions 5 mg. fructose gave an intense 'permanganate' purple colour [Jordan & Pryde, 1938]. On heating at 80° for 15 min. an intense 'permanganate' purple colour was obtained indicative of hexoses. The above qualitative tests indicate the complete absence or presence only in very small amounts of ketose, pentose and uronic acid residues in the polysaccharide molecule, which consists predominantly of hexose units.

Serological properties of polysaccharide

Samples of the *Bact. typhosum* Ty_2 polysaccharide prepared both from the tryptic digest antigen and directly from the dried organisms precipitated with *Bact. typhosum* 'pure' O-antiserum to dilutions of 1 in 300,000 of the polysaccharide; optimal precipitation was obtained at dilutions of 1 in 300,000 and 1 in 150,000 respectively. No precipitation was observed with mixtures of *Bact. typhosum* 'pure' Vi-antiserum and the specific polysaccharide prepared from either source. In this respect the polysaccharide differs markedly from the intact antigenic complex from which it was derived, which precipitated with both O and Vi antisera to dilutions of 1 in 150,000 and 1 in 15,000 respectively, with optimal precipitation at a dilution of 1 in 300 of the antigen in mixtures with O-antiserum. The author is indebted to Dr A. Felix, who kindly carried out the above series of precipitin reactions.

Times of formation of visible precipitates in mixtures of Bact. typhosum *Ty_2 antigen and polysaccharide with O- and Vi-antisera*

	Bact. typhosum Ty_2 'tryptic digest antigen'		*Bact. typhosum* Ty_2 polysaccharide	
	0·5 mg.	0·25 mg.	0·5 mg.	0·25 mg.
'Pure' O-antiserum	+ 10 min.	+ 10 min.	+ 5 min.	+ 3 min.
'Pure' Vi-antiserum	+ 15 min.	+ 15 min.	– ve	– ve

Similar results were obtained in a number of experiments by Dr Joyce Wilson, who observed the times of formation of a visible precipitate when given quantities of polysaccharide and of antigen were mixed with equal volumes of antisera, diluted 1 in 10.

The polysaccharide, which reacts specifically with O-antiserum but not with Vi-antiserum is to be regarded as the O-specific hapten of *Bact. typhosum*.

Homogeneity of polysaccharide

Before undertaking a detailed examination of the nature and properties of the specific polysaccharide it was submitted to a further fractionation with alcohol to determine whether it was possible by this means to separate the material into components which were chemically or serologically distinct. A sample was also examined in the ultracentrifuge for any evidence of more than a single component.

Alcoholic fractionation. A sample (1·84 g.) of polysaccharide was dissolved in water, 50 ml., giving a clear, colourless solution free from any undissolved residue. Absolute alcohol was added to 50% by weight but no precipitate or opalescence was obtained. When the alcoholic concentration was raised to 55% a small precipitate was obtained which was removed by centrifuging. Further increments of alcohol were added until the material had finally been divided into seven fractions. The bulk of the substance (88%) was precipitated between alcoholic concentrations of 55% and 70%. Only 1% was insoluble in 55% alcohol, and 11% remained soluble when the concentration had reached 70%.

The seven fractions isolated were compared by determination of specific optical rotations and reducing values on hydrolysis with 2*N* HCl for 2 hr. at 100° in a sealed tube (Hagedorn and Jensen), and the results obtained are summarized in Table 4.

Table 4. *Alcoholic fractionation of O-specific hapten*

Fraction	Wt. g.	Yield %	$[\alpha]_D^{19°}$	R.V. on hydrolysis (as glucose (%))
Unfractionated polysaccharide	1·84	—	+114°	—
(1) F 50–55%	0·0216	1·1	+108°	67
(2) F 55–57·6%	0·3987	21·7	+111°	77
(3) F 57·6–59·6%	0·3431	18·6	+112°	79
(4) F 59·6–63·2%	0·5286	28·7	+110°	77
(5) F 63·2–70%	0·3557	19·3	+112°	79
(6) F 70–85%	0·1590	8·6	+113°	69
(7) F 85–99%	0·0463	2·5	+113°	—

(Results uncorrected for ash.)

Fractions of the polysaccharide differing slightly in solubility in alcohol-water mixtures have been shown by specific rotation and reducing value (on hydrolysis) determinations to be practically identical and it may be concluded that the O-polysaccharide hapten prepared as described above represents a chemical entity.

Ultracentrifuge data. The above conclusions were confirmed by examination of a sample of the polysaccharide in the ultracentrifuge by J. St L. Philpot, who reports that the substance appeared to be practically homogeneous, 79% being accounted for as a single molecular entity (see Addendum to this paper). The minimum molecular weight is of the order of 10,000, i.e. about 60 anhydrohexose units, and the polysaccharide molecules are probably not spherical.

Acetylation of polysaccharide. A number of preliminary experiments were made on crude samples of the specific polysaccharide to determine the optimal conditions for acetylation. 0·507 g. of the pure substance was dissolved in water (2 ml.) and the solution treated with pyridine, redistilled over KOH, B.P. 115° (25 ml.), and acetic anhydride (25 ml.), the latter being added gradually so that the temperature of the mixture did not

exceed 35° [Challinor *et al.* 1934]. The clear, pale yellow mixture was kept at room temperature for 48 hr., and then poured into ice-cold water (400 ml.) giving an immediate colourless precipitate of acetyl polysaccharide. The crude product was separated off, washed with ice-cold water until substantially free from acetic acid, and dried *in vacuo* over phosphorus pentoxide, giving 0·477 g. crude material (94 %).

The crude acetyl derivative was extracted with about 100 ml. boiling absolute ethyl alcohol in which it dissolved and was thrown down again on cooling. The cooled mixture was kept at 0° overnight and the reprecipitated acetyl polysaccharide centrifuged off and the process repeated. The alcohol-extracted residue was taken up in 15 ml. chloroform and the solution heated on the water bath with a little animal charcoal for about 15 min. The charcoal was removed by centrifuging and the clear supernatant solution treated with excess (6 vol.) of light petroleum B.P. 40–60°. The precipitated acetyl derivative was again taken up in chloroform (15 ml.) and reprecipitated with light petroleum. By addition first of light petroleum to 48 % by weight (32 ml.) followed by centrifuging and raising of the petroleum concentration to 70 % conc. by weight (82 ml.) and again removal of the precipitate by centrifuging, fractions of the acetylated polysaccharide were obtained and analysis showed that the derivative was homogeneous.

The acetyl polysaccharide was a colourless amorphous solid; it was insoluble in cold ethyl alcohol, ether and water, but was readily soluble in chloroform, pyridine and acetone; $[\alpha]_D^{20°}$, +94–96° (in chloroform, $c=1$); organic P, 0·3 %; C, 50·8; H, 5·8; acetyl, 40·0 %. $C_6H_7O_5(OC.CH_3)_3$ requires C, 50·0; H, 5·6; $CH_3.CO$, 44·8 %. Pyridine solutions of the acetyl polysaccharide did not precipitate with *Bact. typhosum* O-antiserum and chloroform solutions of the substance gave no specific precipitation at the interface when O-antiserum was layered upon the chloroform solutions. It is concluded that the polysaccharide hapten loses its property of specifically reacting with the homologous antibody on conversion into the triacetyl compound.

Regeneration of the polysaccharide. After a series of preliminary experiments in which the acetyl polysaccharide dissolved in acetone or pyridine was treated with *N* KOH for various periods of time at 37°, 50°, etc., a product identical with the original specific polysaccharide prepared from the tryptic digest antigen was obtained. Intermediate acetyl derivatives containing 15 and 5·3 % acetyl have also been isolated. In a typical saponification of the acetyl polysaccharide, 0·27 g. of the substance dissolved in 8 ml. pyridine was treated with 2 ml. *N* KOH at 37° for 4 hr. At the end of this period the mixture was diluted with an equal volume of water and dialysed in a cellophane sac for 2 hr. to eliminate the bulk of the excess alkali and pyridine. The dialysed solution was acidified by addition of 2 ml. *N* acetic acid and poured into absolute alcohol (10 vol.) to precipitate the regenerated polysaccharide. The precipitated polysaccharide was taken up in water (10 ml.) and three times fractionated between the limits of 50 and 86 % concentrations of alcohol by weight. Finally the precipitated polysaccharide was washed twice with absolute ethyl alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide; obtained 0·12 g. or 44 % yield; acetyl, 14·9 %.

The regenerated polysaccharide and the partially deacetylated products give precipitates with *Bact. typhosum* O-antiserum to high dilutions, which however differ according to the acetyl content, but no precipitates were obtained with Vi-antiserum. The properties of several partially acetylated polysaccharides are summarized (Table 5) together with details of the conditions of their preparation.

The intermediate acetyl derivatives containing 15 and 5·3 % acetyl resemble the polysaccharide rather than the fully acetylated compound; they are freely soluble in water and precipitate with homologous antiserum in contrast to the acetyl polysaccharide. Loss of acetyl groups is accompanied by a gradual rise in specific optical rotation from +95° to +122°. The precipitation titres of the partially acetylated polysaccharides increase with decreasing acetyl content until a region of maximum precipitability is

Table 5. *Properties of acetylated derivatives of O-specific polysaccharide*

	Method of preparation	C %	H %	Non-volatile ash %	Acetyl %	P %	$[\alpha]_D$	Limiting dilution of precipitation with O-antiserum
I. Fully acetylated polysaccharide: insoluble in water	Acetylation of polysaccharide as described	50·8	5·8	—	40	0·3	+ 95° (18° C.)	Does not precipitate
II. Partially acetylated polysaccharide: water-soluble	0·27 g. I in 8 ml. pyridine + 2 ml. *N* KOH at 37° for 4 hr. Obtained 0·12 g. II (44%)	45·9	6·7	Nil	15	—	+110° (18° C.)	1 in 2500
III. Polysaccharide: water-soluble	2 g. I in 50 ml. pyridine + 50 ml. acetone + 50 ml. water heated with 20 ml. *N* KOH at 50° for 3/4 hr., gave 0·6 g. III (30%)	43·8	6·9	1·9	5·3	0·4	+114° (15° C.)	1 in 125,000
IV. Specific polysaccharide	From 'tryptic digest antigen' as described	44·41	7·12	2·4	3·5	0·4	+115° (16° C.)	1 in 125,000
V. Polysaccharide: water-soluble	0·44 g. III in 20 ml. water + 20 ml. *N* KOH at 50° for 2 hr. obtained 0·318 g. V (73%)	—	—	—	2·1*	—	+122° (17° C.)	1 in 125,000
VI. Deacetylated polysaccharide: water-soluble	0·44 g. I in 20 ml. pyridine + 20 ml. water heated with 20 ml. *N* KOH at 100° for 1 hr. gave 0·144 g. VI (33%)	43·9	6·8	2·2	Nil	—	+121° (23° C.)	1 in 125,000

* N = 0·6%. Results uncorrected for ash.

reached at acetyl contents of 0–5%. Polysaccharides III and V are practically identical, in chemical properties and serological precipitation, with the original specific polysaccharide, IV, prepared from the 'tryptic digest antigen'. This affords further strong evidence that the specific properties of the latter are a function of the polysaccharide and not of small amounts of concomitant impurities [cf. Heidelberger & Avery, 1924].

Benzoylation of the polysaccharide

0·5 g. of the polysaccharide was dissolved in 20 ml. water; benzoyl chloride (2·5 ml.) was gradually added with vigorous shaking the reaction mixture being kept alkaline by addition of 2*N* NaOH. The benzoylated polysaccharide separated almost immediately from the solution as a heavy, white precipitate. This was filtered off, washed successively with 0·01*N* NaOH, water and alcohol and dried *in vacuo* over phosphorus pentoxide. The crude benzoyl polysaccharide (0·70 g.) was insoluble in ether, slightly soluble in chloroform and acetone, but freely soluble in pyridine. The crude material was dissolved in about 5 ml. of pyridine and reprecipitated by addition of excess ether, after the pyridine solution had been centrifuged to remove traces of insoluble matter. This process was twice repeated and the benzoyl derivative finally washed with ether until free from pyridine and dried *in vacuo* over phosphorus pentoxide. The benzoylated polysaccharide was a light cream-coloured amorphous solid, $[\alpha]_D^{16\cdot5°}$, +93° (in pyridine, $c = 1$); organic P, 0·4; C, 59·2; H, 5·2; non-volatile ash, 1·6%.

Acid hydrolysis of the specific polysaccharide

The optimal conditions of liberation of reducing sugars by hydrolysis of the polysaccharide with mineral acid were investigated as a preliminary to identification of the sugars present. 0·0143 g. of the polysaccharide (dried *in vacuo* at 100° for 1 hr.) was dissolved in 25 ml. *N* H_2SO_4 and the clear solution hydrolysed under reflux on the boiling water bath. 1 ml. samples were withdrawn at intervals, neutralized and the reducing sugars determined by the Hagedorn and Jensen method. A determination was also made on the unhydrolysed polysaccharide (5 mg.) in 2 ml. water. The results (Table 6)

Table 6. *Liberation of reducing groups from the polysaccharide by hydrolysis with* N H_2SO_4

Time hr.	Apparent glucose liberated mg.	Reducing value %	Time hr.	Apparent glucose liberated mg.	Reducing value %
0*	0·054	1·1	3·5	0·219	76·6
0†	0·024	8·5	5	0·225	78·7
0·5	0·128	45·0	6	0·226	79·0
1	0·169	59·3	8	0·223	78·0
2	0·206	72·0			

* From 4·95 mg. polysaccharide in neutral solution.
† In *N* H_2SO_4 solution.

show that hydrolysis proceeds rapidly for the first hour and then becomes markedly slower until after 5–6 hr. the liberation of reducing groups reaches a maximum of 79 % and thereafter falls gradually owing to destruction of the liberated sugars. The addition of *N* H_2SO_4 (which was shown to be free from reducing substances) produced an appreciable increase (7 %) of reducing power in the cold; this may be due to an effect on associated polysaccharide chains but is not fully understood. The author is indebted to Dr T. H. Anderson for the above reducing value determinations.

Nature of component reducing sugars

Evidence that the specific polysaccharide consists predominantly of anhydrohexose units has already been described. The presence of *d*-galactose in the antigenic complex from which the polysaccharide was derived has been demonstrated by Freeman *et al.* [1940]. The presence of this sugar in the specific polysaccharide was therefore sought at the outset; it was, however, necessary to test for the presence of *d*-mannose as the latter, if present, forms an insoluble methylphenylhydrazone under the optimal conditions of precipitation of galactose methylphenylhydrazone [Müther & Tollens, 1904].

0·4–0·6 g. quantities of the dried specific polysaccharide were hydrolysed by heating on the water bath at 100° for 5 hr. with 60 ml. *N* H_2SO_4. The acid was removed from the cooled solution after hydrolysis by addition of a slight excess of hot saturated baryta to the hydrolysate cooled in ice, the precipitated Ba salts were removed by centrifuging and the supernatant solution and washings made just acid by addition of 2*N* H_2SO_4 and the excess acid removed by addition of an excess of pure $BaCO_3$. The clear, colourless, neutral filtrate was concentrated *in vacuo* at low temperature to 10–20 ml. bulk. The optical rotation and reducing value (Hagedorn and Jensen) of the solution were determined at this stage: $\alpha_D^{16°} = +0·85°$, 1 dm. (volume of solution, 17·8 ml.); total apparent glucose = 0·3066 g. (or 75 % of the original polysaccharide). On the assumption that the reducing constituents of the solution have a reducing value approximating to that of glucose, the 'apparent specific rotation' of the contents of the solution is $[\alpha]_D^{16°} = +49°$. (Specific rotation, $[\alpha]_D$, glucose, +52·8°; galactose, +81°; and mannose, +14·2°.)

d-*Mannose phenylhydrazone*. The presence of *d*-mannose in the polysaccharide hydrolysate was proved by the formation of *d*-mannose phenylhydrazone on treatment with phenylhydrazine in acetic acid solution. The neutral hydrolysate corresponding to 0·385 g. specific polysaccharide (25 ml. volume) was treated with a mixture of 0·2 g. freshly distilled phenylhydrazine (B.P. 120–124°/15 mm.) and 0·4 g. of 25 % acetic acid [van der Haar, 1920]. The mixture was allowed to crystallize overnight at 0° giving a mass of crystals of *d*-mannose phenylhydrazone, which were filtered off, washed with a little ice-cold water, then with ice-cold alcohol and ether and dried *in vacuo* over phosphorus pentoxide. The mother liquor and aqueous washings were concentrated to 9 ml. and treated with 0·1 g. phenylhydrazine and 0·2 g. 25 % acetic acid when a further quantity of crystals appeared after 24 hr. at 0°. The crude product was recrystallized from boiling 60 % alcohol : 40 % water mixtures to constant M.P.; *d*-mannose phenylhydrazone, colourless microcrystalline plates, M.P. 193–195°, unchanged on admixture with an authentic specimen was obtained. (Found: C, 53·3; H, 6·7; N, 10·3 %. $C_{12}H_{18}N_2O_5$ requires C, 53·3; H, 6·7; N, 10·4 %.) The yield of crude material was 0·1156 g., corresponding to a mannose content of 20·0 % of the specific polysaccharide.

The excess phenylhydrazine was removed as benzaldehyde phenylhydrazone [Morgan, 1938]. Treatment with benzaldehyde also liberates any hexoses that have formed soluble phenylhydrazones. The mother liquors and aqueous washings from the mannose phenylhydrazone crystallizations were concentrated *in vacuo* to 15 ml. and heated with 0·8 g. freshly distilled benzaldehyde and 0·02 g. benzoic acid for 1 hr. under reflux on the boiling water bath in an atmosphere of CO_2. The mixture was then cooled to 0°, filtered and the oily residue washed twice with small quantities of ice-cold water; the combined filtrate and washings were then immediately extracted several times with ether and the slightly coloured aqueous solution concentrated *in vacuo* to about 10 ml. The solution was heated at 100° for about 10 min. with a small quantity of norit, which had previously been extracted with conc. HCl and well washed with water. The almost colourless filtrate containing the remaining sugars was concentrated *in vacuo* to 10 ml.

d-*Galactose methylphenylhydrazone*. The methylphenylhydrazone of *d*-galactose was isolated under the conditions described by Neuberg [1907] and Lüdtke [1929]; the aqueous sugar-containing solution was treated with an equal volume of absolute ethyl alcohol, 0·2 g. methylphenylhydrazine (asym.), redistilled B.P. 104° at 14 mm., and 0·1 g. 50 % acetic acid. The mixture was kept at 37° for 7 hr. and then at 0° for a further 17 hr. Galactose methylphenylhydrazone crystallized as colourless, microcrystalline plates which were filtered off on to a small filter paper in a tared Gooch crucible, washed with 10 ml. ice-cold absolute alcohol and dried to constant weight *in vacuo* over phosphorus pentoxide. The mother liquors and washings were concentrated *in vacuo* at low temperature to 2–3 ml. volume, 10 ml. absolute methyl alcohol added and the mixture kept at 0° for 24 hr.; usually no further crystallization of the hydrazone occurred. The crude product was recrystallized from hot 50 % pyridine : 50 % ethyl alcohol mixtures; after 1–2 crystallizations pure *d*-galactose methylphenylhydrazone, M.P. 190°, was obtained. The M.P. was unchanged on admixture with authentic *d*-galactose methylphenylhydrazone (M.P. 190°). (Found: C, 55·1; H, 7·2; N, 10·2 %. $C_{13}H_{20}N_2O_5$ requires C, 54·9; H, 7·1; N, 9·9 %.) The yield of crude hydrazone from 0·385 g. specific polysaccharide was 0·0688 g., or 11·3 % galactose in terms of the original polysaccharide.

Excess methylphenylhydrazine was eliminated as described above for phenylhydrazine and the aqueous solution concentrated *in vacuo* at low temperature to 10 ml. At this stage the solution had $\alpha_D^{19°}$, +0·33°, 1 dm., equivalent to 0·0625 g. apparent glucose in 10 ml.

Glucosazone. The aqueous solution was treated with 0·5 g. phenylhydrazine hydrochloride (decolorized with norit and recrystallized) and 0·75 g. sodium acetate crystals

and the pale yellow solution heated at 100° on the water bath for 1 hr. Glucosazone crystals appeared after about 30 min. in the hot solution, and crystallization was complete after 17 hr. at 0°. The osazone was filtered off, washed with 2–4 ml. ice-cold water, a few ml. cold alcohol and finally with ether and dried *in vacuo* over phosphorus pentoxide. The yield of crude phenylglucosazone was 0·0174 g. (0·1 g. pure glucose treated with phenylhydrazine HCl and sodium acetate under the conditions described gave 0·033 g. glucosazone). The crude product was recrystallized several times from dilute aqueous ethyl alcohol finally giving an osazone which had a typical wheatsheaf microscopical appearance and M.P. 203°; on admixture with an authentic specimen of glucosazone (M.P. 205°) gave mixed M.P. 203°. The identity of the osazone was confirmed by determination of its optical rotation in alcoholic pyridine solution, and comparison with that of an authentic specimen [Neuberg, 1899].

d-*Glucose* p-*nitrophenylhydrazone.* The presence of glucose as a unit in the molecule of the specific polysaccharide was confirmed and its amounts determined by isolation of the sugar as the *p*-nitrophenylhydrazone from a further batch of neutralized polysaccharide hydrolysate [van Ekenstein & Blanksma, 1903; Reclaire, 1908]. The neutral aqueous solution equivalent to 0·5887 g. of specific polysaccharide was concentrated *in vacuo* at low temperature to a syrup which was taken up in methyl alcohol (5 ml.), and the solution heated with 0·6 g. pure *p*-nitrophenylhydrazine at 100° on the water bath under reflux for 10 min. After 24–48 hr. at 0° a yellow, crystalline mass of *d*-glucose *p*-nitrophenylhydrazone had separated; the product was filtered off, washed with ice-cold alcohol and with ether and dried *in vacuo* over phosphorus pentoxide. The mother liquors and washings were evaporated almost to dryness *in vacuo*, diluted to 2–3 ml. with methyl alcohol and allowed to crystallize at 0°; further crops of hydrazone were obtained in this way. The total yield of crude *p*-nitrophenylhydrazone was 0·3338 g., equivalent to a glucose content of 32·4 % of the specific polysaccharide. The hydrazone was recrystallized from hot ethyl alcohol until a constant M.P. of 184° was obtained. The mixed M.P. with an authentic specimen of *d*-glucose *p*-nitrophenylhydrazone (M.P. 184°) was 183°. (Found: C, 45·9; H, 5·5; N, 13·2 %. $C_{12}H_{17}O_7N_3$ requires C, 45·7; H, 5·4; N, 13·3 %.)

The method of identification of the constituent hexose units of the specific polysaccharide as described above, viz. treatment of the neutralized polysaccharide hydrolysate with substituted phenylhydrazines under conditions in which the derivative of a single hexose is obtained at each stage, provides conclusive proof of the co-existence of *d*-glucose, *d*-mannose and *d*-galactose as constituents of the specific polysaccharide. The losses of sugars in the intermediate removal of excess of substituted hydrazines with benzaldehyde are, however, sufficiently great markedly to lower the yields of hydrazones of the sugars. Thus when mannose, galactose and glucose derivatives were isolated in that order from the same polysaccharide hydrolysate the yields of galactose and glucose derivatives were low. In order to obtain a more accurate determination of the quantities of these hexoses present in the original specific polysaccharide it is therefore necessary to take aliquot portions of a neutralized hydrolysate and treat with the appropriate substituted hydrazines. Under these conditions yields of substituted hydrazones equivalent to 32·4 % glucose, 20·0 % mannose and 15·8 % galactose have been obtained. Control experiments were made under the same conditions with similar concentrations of single pure hexoses, when pure *d*-glucose gave 79·6 % of the theoretical yield of the *p*-nitrophenylhydrazone, pure mannose gave 94·9 % of the theoretical yield of *d*-mannose phenylhydrazone and pure galactose gave 90–95 % of the theoretical quantity of *d*-galactose methylphenylhydrazone (with larger quantities of hexose in more concentrated solutions the yields of hydrazones are practically theoretical). On this basis a rough estimate of the actual hexose contents of the specific polysaccharide has been made (Table 7).

Table 7. *Hexose contents of* Bact. typhosum Ty_2 *specific polysaccharide*

	Observed yield %	Yield from pure hexose under identical conditions %	Estimated content of polysaccharide %
d-Glucose	32·4	80	40
d-Mannose	20·0	95	21
d-Galactose	15·8	90–95	17
Total hexose	68·2		78

The total hexose thus accounted for, i.e. 78 %, agrees well with the reducing value of 80–83 % in terms of apparent glucose, obtained when 5 mg. samples of the substance are hydrolysed with 2 ml. quantities of 2 *N* HCl in sealed tubes for 2 hr. at 100° (Hagedorn and Jensen). If the specific polysaccharide consisted entirely of anhydrohexose units it would yield about 111 % apparent glucose on complete hydrolysis. Of the 30 % so far unaccounted for, 4–5 % is due to the presence of acetyl groups and the remainder probably consists of non-reducing substances not yet identified. Decomposition of hexose during acidic hydrolysis may account for some of the undetermined fraction; it is unlikely that an appreciable quantity of the polysaccharide remains unhydrolysed under these conditions as the neutralized hydrolysate gives no appreciable precipitate on addition of 10 vol. of absolute alcohol, which completely precipitate the untreated polysaccharide.

Acid equivalent of specific polysaccharide

A sample of the specific polysaccharide (0·78 g.) was three times reprecipitated between the limits of alcoholic concentration of 50 and 86 % by weight, with acid-free alcohol redistilled over KOH. The reprecipitated polysaccharide (0·713 g.) was dried at 100° and 1–2 mm. for 3 hr., dissolved in 10 ml. water and titrated with 0·01 *N* NaOH (CO_2-free) using phenolphthalein as indicator. The polysaccharide required 2·30 ml. 0·0093 *N* NaOH for neutralization giving an acid equivalent of 30,000.

Action of enzymes

The stability of the polysaccharide towards enzymes capable of splitting α-glucosidic linkages in polysaccharides, viz. α- and β-malt amylases and β-linkages in glucosides (emulsin) was investigated. Malt amylase was used as a source of the mixed α- and β-malt amylases and as β-glucosidase, a commercial sample of emulsin which had been fractionated to remove a part of the inactive proteins, was used. A sample of the specific polysaccharide incubated at 25° and *p*H 4·63 with malt amylase showed no appreciable liberation of reducing sugars after 24 hr. at which time a parallel conversion of soluble starch with the same enzymic concentration had reached 79 % hydrolysis as apparent maltose. The polysaccharide-emulsin mixtures were incubated at 25° and *p*H 5·0; there was no detectable liberation of reducing sugars. A parallel emulsin hydrolysis of β-methyl glucoside under the same conditions produced 21·7 % hydrolysis after 23 hr. and 76·3 % after 168 hr.

Discussion

A specific polysaccharide associated with the O-antigen of *Bact. typhosum* has been isolated both directly from the dried bacterial cells and from the soluble antigenic complex of the organism, which has been shown by earlier workers [Raistrick & Topley, 1934; Topley *et al.* 1937] to account for the specific properties of the intact organism. The complete antigenic complex comprises besides the specific polysaccharide, an insoluble polypeptide component, a soluble nitrogenous substance and a small amount of lipid constituents [Freeman & Anderson, 1941], but of these the polysaccharide is probably of outstanding importance and accounts for some, if not all, of the specific properties of the O-antigen.

Although the specific substance precipitates to extreme dilution with *Bact. typhosum* O-antiserum it has not yet been determined whether it accounts for all the specific serological properties of the O-component of the antigenic complex, but work on these lines is proceeding. The polysaccharide probably plays the part of a hapten as defined by Landsteiner, but it has not yet been possible to determine whether or not it is capable of exhibiting antigenic properties.

The O-specific substance belongs to the class of polysaccharides and exists as a colloidal compound of high molecular weight. The usual chemical criteria of purity cannot be applied in determining its integrity; several attempts to divide it into chemically distinct components by means of fractionation from aqueous solution by means of solvents such as ethyl alcohol and acetic acid have so far failed to produce any components which differed significantly in chemical composition and properties. This evidence, coupled with the facts that the polysaccharide behaves as a single molecular entity in the ultracentrifuge and that it may be reconstituted in a virtually unchanged form from its triacetyl derivative, provides reasonable justification for the claim that it is a single substance. The possibility of admixture with non-specific polysaccharides derived from agar or other constituents of the culture medium was eliminated by the use of a 'synthetic' medium for the growth of the organisms from which the specific polysaccharide was derived.

Soru & Combiesco [1940] have isolated a polysaccharide by dilute acetic acid hydrolysis of the O-antigen of *Bact. typhosum*; this substance has apparently not been submitted to extensive fractionation and its state of purity is unknown but its chemical composition shows that it is probably identical with the specific polysaccharide described in the present communication. Soru & Combiesco's polysaccharide is nitrogen-free, gives 72·4 % of reducing sugars on hydrolysis, is highly dextrorotatory ($[\alpha]+125°$) and contains 11·65 % of ash and 2·88 % of P (results, except that for P, calculated on an ash-free basis) as compared with the present polysaccharide which contains 0·2–0·4 % N, gives 83 % of reducing sugars on hydrolysis, has a specific rotation of $[\alpha]_D^{16°}$ +114–115° and contains about 2 % of ash and 0·4–0·9 % P. It is not yet clear whether the small nitrogen content of our polysaccharide is due to a small quantity of impurity or whether the nitrogen is an essential component of the polysaccharide. The latter alternative would appear to be more probable as a specifically reacting polysaccharide containing 0·6 % N has been reconstituted from the triacetyl derivative (Table 5). Many of the polysaccharides isolated from bacteria during recent years as well as others which have some specific biological role have been found to contain *N*-acetylhexosamine as an important unit of the polysaccharide molecule, e.g. the polysaccharide hapten of smooth forms of *B. dysenteriae* (Shiga) [Jolles & Morgan, 1940], the specific polysaccharide of *Pneumococcus* Type IV [Heidelberger & Kendall, 1931], Freudenberg & Eichel's [1934], Group A polysaccharide from urine, and the carbohydrate of ovomucoid [Stacey & Woolley, 1940]. On the other hand, the important acetyl polysaccharide of *Pneumococcus* Type I [Avery & Goebel, 1933] contains an unacetylated amino-sugar, the acetyl groups being attached through oxygen directly to a carbon atom. In the present polysaccharide some of the acetyl groups are probably attached to nitrogen and some are in the form of O-acetyl, but in the case of polysaccharide V (Table 5) obtained by partial deacetylation of the fully acetylated derivative, the acetyl groups are probably attached to nitrogen atoms in view of the relatively vigorous conditions required to eliminate them. This interesting problem of the mode of linkage of the acetyl groups in the polysaccharide is receiving further investigation.

The bulk of the constituent reducing sugars which comprise the polysaccharide molecule has been identified and isolated as substituted phenylhydrazones. 40 % *d*-Glucose has been isolated as the *p*-nitrophenylhydrazone, 21 % of *d*-mannose as mannose phenylhydrazone and 17 % *d*-galactose as the methylphenylhydrazone. That it has been possible

to separate the hexoses in this way depends upon the fact that mannose is the only hexose which readily gives an insoluble phenylhydrazone under the experimental conditions. Thus *d*-galactose also forms a phenylhydrazone but only when a very concentrated aqueous solution of the sugar is treated with phenylhydrazine. Most of the hexoses, including mannose, galactose and glucose form *p*-nitrophenylhydrazones under suitable conditions but that of *d*-glucose is the only one which readily crystallizes under the conditions of our experiments [van Ekenstein & Blanksma, 1903; Reclaire, 1908; van der Haar, 1920]. The *p*-nitrophenylhydrazone isolated in this investigation melted at the same temperature, 183–184°, alone or mixed with an authentic sample of *d*-glucose *p*-nitrophenylhydrazone. (The *p*-nitrophenylhydrazones of the hexoses exist in two isomeric forms according to whether they crystallize from acetic acid or alcoholic solutions [van Ekenstein & Blanksma], the lower-melting form from alcoholic solution was isolated in this work.) As a further confirmation of the presence of *d*-glucose in the hydrolysates from the specific polysaccharide, phenylglucosazone was isolated from a hydrolysate from which the mannose had been carefully and completely removed, and in a yield of the same order as that obtained from pure glucose at the same concentration. Glucose, fructose, xylose and glucuronic acid do not interfere with the determination of *d*-galactose by means of methylphenylhydrazine; arabinose, mannose and fucose, according to Müther & Tollens [1904] are precipitated under the same conditions as galactose. The intervention of mannose was avoided by prior precipitation of this sugar as the phenylhydrazone. A useful confirmation of the relative proportions of the three hexoses present in the polysaccharide hydrolysates is afforded by a comparison of the observed optical rotation of a neutralized hydrolysate with that calculated from the reducing value of the solution (Hagedorn and Jensen) in terms of apparent glucose. The hydrolysate described above had $\alpha_D^{16^\circ} = +0{\cdot}85^\circ$ (1 dm.), total volume 17·8 ml. and total apparent glucose, 0·3066 g. (Hagedorn and Jensen). In such a solution containing glucose, mannose and galactose in the ratio of 40 : 21 : 17, the rotation of the *d*-glucose component is +0·47°, that of the mannose component +0·07° and that of the galactose +0·30° giving a total calculated rotation, 1 dm., of +0·84°, which agrees sufficiently accurately with the observed rotation.

The observation of Goebel [1939; 1940] that relatively simple carbohydrate molecules such as those of aldobionic acids may have specific properties in relation to bacterial antigens, e.g. by specifically inhibiting precipitation between antigen or hapten and homologous antiserum if the latter be initially incubated with an aldobionic acid derived from the complex polysaccharide hapten, or by determining the specificity of artificial carbohydrate-protein antigens, has stimulated search for a simpler pattern unit in the specific carbohydrate of *Bact. typhosum*. Attempts to isolate such a simpler specific carbohydrate unit by graded hydrolysis of the polysaccharide hapten have so far proved unsuccessful.

Summary

1. A method for the extraction and purification of an O-specific polysaccharide from the bacterial cells or isolated antigenic complex of *Bact. typhosum* Ty_2 has been described.

2. The polysaccharide is dextrorotatory, $[\alpha]_D^{16^\circ} = +114\text{–}115^\circ$; $[\alpha]_{5461}^{16^\circ} = +135^\circ$, it contains 0·2–0·4 % nitrogen, and 0·4–0·9 % phosphorus, and yields 83 % of reducing sugars on acidic hydrolysis.

3. The polysaccharide appears to be free from ketose, pentose and uronic acid residues but yields 40 % *d*-glucose, 21 % *d*-mannose and 17 % *d*-galactose on hydrolysis.

4. The specific substance precipitates to a high dilution with *Bact. typhosum* O-antiserum but not with Vi-antiserum. Elimination of the 3 % of acetyl groups in the molecule does not appear to influence the limit of precipitation with O-antiserum.

5. The polysaccharide has a minimum molecular weight of the order of 10,000, i.e. about 60 anhydrohexose units.

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Examination in the Ultracentrifuge

By J. St L. Philpot

The specimen of polysaccharide was examined in the Svedberg ultracentrifuge, the conditions being: concentration, 1%; *p*H, 7; NaCl concentration, 0·2 *M*; centrifugal field, 300,000 times gravity. Fig. 1 shows a photograph by the 'diagonal schlieren' method about 2 hr. after the start of sedimentation. The corrected sedimentation constant is $1{\cdot}2 \times 10^{-13}$, which corresponds to a molecular weight of about 10,000 for a spherical

molecule, or more for an aspherical one. The maximum in Fig. 1 is sharper than it would be with a spherical molecule of weight 10,000, so the heavier aspherical molecule is more

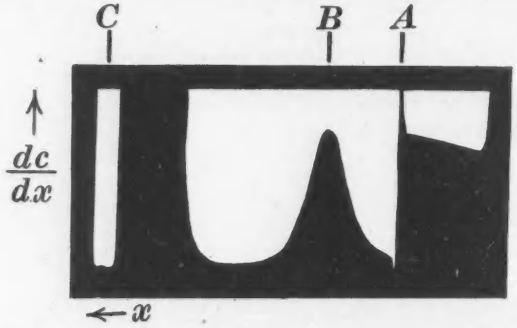

Fig. 1. Sedimentation curve of *Bact. typhosum* polysaccharide. x = distance from centre of rotation (arbitrary units). dc/dx = concentration gradient (arbitrary units). A = meniscus, B = sedimentation boundary, C = index.

likely. The refractive increment of the 1 % solution was 0·00127, and 79 % of this value was obtained by integration of the refractive gradient curve in Fig. 1. Hence 79 % ± about 5 % of the material is homogeneous in sedimentation.

39. Analogues of Pantothenic Acid

1. Attempted Preparation of Growth Promoters

By **Jean W. Barnett** and **F. Arnold Robinson**, *Chemical Research Department, Glaxo Laboratories Ltd., Greenford, Middlesex*

(*Received 25 March 1942*)

In view of the importance of pantothenic acid as a growth factor for certain micro-organisms, it was decided to investigate the specificity of this compound by preparing analogues in which one or more groups were omitted from or introduced into the molecule and testing the effect of such constitutional variations on the biological activity. The present paper deals with the effect of such variations on growth-promoting activity, whilst the following paper describes the preparation of analogues of pantothenic acid which were growth inhibitors. Further details of the biological testing and a fuller discussion of the results will be published by Dr H. McIlwain who carried out the biological work.

A number of analogues of pantothenic acid have already been described in the literature, and Table 1 shows their biological activities expressed as fractions of that of pantothenic acid. The compounds were prepared either by condensing β-alanine with various lactones or by condensing amino-acids other than β-alanine with α-hydroxy-$\beta\beta$-dimethyl-γ-butyrolactone.

We have now prepared additional analogues of pantothenic acid as shown in Table 2. Of these nos. 16 and 17 differ from pantothenic acid only in the absence of methyl groups, while no. 13 differs only in the absence of the α-hydroxy group. The lactones involved in the preparation of these three analogues have been prepared previously. Nos. 14 and 15 contain one carbon atom more than pantothenic acid; neither of the lactones corresponding to these two compounds has been described before. Homopantoyllactone has now been made by a Reformatsky condensation on the corresponding aldol, and dehydrohomopantoyllactone by a malonic acid condensation on the same compound in presence of acetic acid. Numbers 18 to 21 are analogues formed by condensing the parent lactone (α-hydroxy-$\beta\beta$-dimethyl-γ-butyrolactone) with amino-acids other than β-alanine.

Table 2 shows the results of growth stimulation tests on these analogues. Nos. 13–17 were tested in the form of their sodium salts, while nos. 18–21 were in the form of their calcium salts. Nos. 13–17 were tested for stimulation of growth on *Proteus morgani*. The maximum activity observed was 10^{-4} of that of pantothenic acid; hence the replacement effect in all five cases was negligible.

The suggestion that certain of the pantothenic acid analogues described in this paper might be of biological interest was made to us by Dr McIlwain, who also suggested a simplified nomenclature, following which we have adopted a system based on the name 'pantoyl' for the α:γ-dihydroxy-β:β-dimethyl-γ-butyryl radicle:

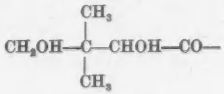

Table 1. *Showing the formulae and biological activities of analogues of pantothenic acid prepared previously*

No.	Amino-acid	Lactone	Formula of analogue	Growth stimulation as a fraction of pantothenic acid activity	Reference
1	β-Alanine	α-Hydroxy-ββ-dimethyl-γ-butyrolactone	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—COOH$	1	
2	,,	α-Hydroxy-γ-valerolactone	$CH_3—CHOH—CHOH—CH_2—CO—NH—CH_2—CH_2—COOH$	0	Reichstein [1940] Mitchell *et al.* [1940, 1]
3	,,	α-Hydroxy-δ-valerolactone	$CH_2OH—CH_2—CH_2—CHOH—CO—NH—CH_2—CH_2—COOH$	0 +ve ?	Reichstein [1940] Subbarow & Rane [1939]
4	,,	α-Hydroxy-α-methyl-γ-butyrolactone	$CH_2OH—CH_2—C(CH_3)OH—CO—NH—CH_2—CH_2—COOH$	+ve, $<10^{-2}$	Mitchell *et al.* [1940, 1]
5	,,	α-Hydroxy-β-methyl-γ-butyrolactone	$CH_2OH—CH(CH_3)—CHOH—CO—NH—CH_2—CH_2—COOH$	+ve, $<10^{-2}$	Mitchell *et al.* [1940, 1]
6	,,	α-β-Dihydroxy-γ-butyrolactone	$CH_2OH—CHOH—CHOH—CO—NH—CH_2—CH_2—COOH$	0	Mitchell *et al.* [1940, 1]
7	,,	α-Hydroxy-γ-butyrolactone	$CH_2OH—CH_2—CHOH—CO—NH—CH_2—CH_2—COOH$	0	Mitchell *et al.* [1940, 1]
8	,,	α-Hydroxy-β-methyl-β-hydroxymethyl-γ-butyrolactone	$CH_2OH—C(CH_3)(CH_2OH)—CHOH—CO—NH—CH_2—CH_2—COOH$	+ve, *c.* 1/5	Mitchell *et al.* [1940, 2]
9	α-Alanine	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH(CH_3)—COOH$	0	Weinstock [1940]
10	β-Amino-butyric acid	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH(CH_3)—CH_2—COOH$	0	Weinstock [1940]
11	Aspartic acid	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH(CH_2—COOH)—COOH$	0	Weinstock [1940]
12	Lysine	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH((CH_2)_4NH_2)—COOH$ (?)	0	Weinstock [1940]

Table 2. *Showing the formulae and biological activities of new analogues of pantothenic acid*

No.	Amino-acid	Lactone	Formula and derived name of analogue	Growth stimulation as a fraction of pantothenic acid activity		
				Prot. morgani	*B. coli*	*Strep.*
1	β-Alanine	α-Hydroxy-ββ-dimethyl-γ-butyrolactone	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—COOH$	1		
13	,,	ββ-Dimethyl-γ-butyrolactone	$CH_2OH—C(CH_3)_2—CH_2—CO—NH—CH_2—CH_2—COOH$ Deoxypantothenic acid	$<10^{-5}$		
14	,,	β-Hydroxy-γγ-dimethyl-δ-valerolactone	$CH_2OH—C(CH_3)_2—CHOH—CH_2—CO—NH—CH_2—CH_2—COOH$ Homopantothenic acid	$<10^{-4}$		
15	,,	Δα:β-γγ-Dimethyl-δ-pentenolactone	$CH_2OH—C(CH_3)_2—CH=CH—CO—NH—CH_2—CH_2—COOH$ Dehydrohomopantothenic acid	$<10^{-5}$		
16	,,	γ-Butyrolactone	$CH_2OH—CH_2—CH_2—CO—NH—CH_2—CH_2—COOH$ *bisnor*Deoxypantothenic acid	$<10^{-5}$		
17	,,	γ-Valerolactone	$CH_3—CHOH—CH_2—CH_2—CO—NH—CH_2—CH_2—COOH$ *isonor*Deoxypantothenic acid	$<10^{-5}$		
18	Lysine	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH((CH_2)_4NH_2)COOH$ (?)	Do not appear to have marked activity		
19	Leucine	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH—(CH_2—CH(CH_3)_2)COOH$			
20	Valine	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH(CH(CH_3)_2)COOH$			
21	Taurine	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—SO_3H$			

Preparation of the analogues

Chemical method

All the methods so far published for the synthesis of pantothenic acid depend on the condensation of α-hydroxy-ββ-dimethyl-γ-butyrolactone (pantolactone), with β-alanine in the form of its esters or salts. This lactone has already been prepared [Glaser, 1904; Köhn & Neustädter, 1918] from the cyanohydrin of αα-dimethyl-β-hydroxypropaldehyde. The aldol itself was described by Wessely [1900] and a modified method of preparation was given by Stiller *et al.* [1940]. Glaser [1904] prepared the cyanohydrin by condensing the aldol with liquid HCN, whilst Stiller *et al.* [1940] prepared it by treating the bisulphite compound of the aldol with potassium cyanide. An improvement on the original method was described by Reichstein [1940], who used sodium cyanide and calcium chloride instead of liquid HCN, the hydrolysis being effected with alkali. A further simplification was published by Carter & Ney [1941] who used sodium cyanide and calcium chloride but hydrolysed the cyanohydrin by means of the calcium hydroxide produced during the reaction. We have found this simplified method satisfactory.

The condensation with β-alanine was effected originally through the ethyl ester of the amino-acid [Stiller *et al.* 1940], whilst a more direct method involving fusion at 100° of the lactone and β-alanine sodium salt was published about the same time by Williams *et al.* [1940]. A simpler technique was described by Reichstein [1940] who found that condensation took place when the lactone and the methyl ester of β-alanine were refluxed together in absolute methyl alcoholic solution for 1 hr.; on removing the solvent and hydrolysing the residue carefully, a solution of pantothenic acid was obtained which was tested directly.

We adapted this last method to the preparation of the sodium and calcium salts of pantothenic acid and its analogues, and found that complete or nearly complete condensation took place when the calcium or sodium salt of β-alanine, or other amino-acid, was refluxed for 1 hr. with the appropriate lactone in absolute methyl alcohol (dried over barium oxide and freshly redistilled before use) with exclusion of moisture. A practically clear solution was obtained which, after being filtered quickly from traces of insoluble material, was poured into about 6 vol. of absolute ether or acetone with vigorous shaking. This gave a fine white precipitate, which was filtered off and washed with absolute ether. Owing to the extremely hygroscopic nature of the sodium salts of most of the analogues, it was necessary to keep the precipitate covered with ether all the time, finally transferring it to a desiccator over phosphorus pentoxide and removing the ether *in vacuo*; the substance was left as a dry white amorphous powder. Synthetic pantothenic acid prepared by this method was fully active when tested bacteriologically. A similar amorphous product could be obtained by evaporating the alcoholic solution to small bulk and cooling. Substantially the same method was described recently by Parke & Lawson [1941], using ethyl alcohol as solvent.

Crystalline sodium pantothenate was obtained by Reichstein [1940] who found that condensation could be effected by adding one equivalent of sodium methoxide to β-alanine in methyl alcohol, followed by the addition of the lactone to the cooled solution, and keeping the mixture at room temperature for 2 days. Concentration of the solution followed by careful addition of acetone gave the crystalline product.

In the normal way, the efficiency of the condensation of the two components to form pantothenic acid, is tested biologically. (In our experiments with pantothenic acid itself, full activity was observed with the sodium salt, prepared both by the fusion method of Williams and by the refluxing method just described.) In the case of the analogues, however, obviously the biological method could not be used to test complete condensation.

We therefore carried out total N determinations on all the analogues. It is clear that if incomplete condensation had occurred, then the ether precipitation would throw down the analogue together with all the amino-acid sodium salt, but would probably not throw out the unchanged lactone, or at any rate only partially. A high total N may therefore be taken to indicate incomplete condensation. We have taken the total N as a rough indication of the maximum condensation which has taken place. The values in every case indicated over 80 % conversion. In only one case (homopantothenic acid) was a nitrogen value found which was slightly lower than the theoretical, but the difference was practically within the limits of experimental error.

Deoxypantothenic acid (no. 13)

ββ-Dimethylbutyrolactone. The lactone was prepared according to the method of Windaus [1921] by decomposition of the silver salt of ββ-dimethylglutaric acid [Kommpa, 1899]. The yield obtained was lower than that given by Windaus (27 % instead of 40 % at the final stage), 17 g. of silver salt giving 1·5 g. of crude lactone, from which 0·9 g. of pure lactone was obtained after two sublimations. It is a hygroscopic, very volatile, waxy solid; M.P. 57° (lit. 57°). The amide, although crystalline, proved to be too deliquescent to be of use for analysis. (Found: C, 62·5, H, 8·6 %. Calc. for $C_6H_{10}O_2$: C, 63·1; H, 8·8 %.)

Condensation. Freshly resublimed lactone (553 mg.) and 542 mg. of β-alanine sodium salt were dissolved in 4 ml. of absolute methyl alcohol. The solution was refluxed for 2 hr. and worked up as described for the general procedure. Yield, 840 mg. (Found: N, 7·1 %. Calc. for $C_9H_{16}O_4N$ Na: N, 6·3 %.) This may be taken to indicate 88 % condensation.

Homopantothenic acid (no. 14)

β-Hydroxy-γγ-dimethyl-δ-valerolactone. 50 g. of αα-dimethyl-β-hydroxy-propaldehyde (B.P. 90–92°/121 mm.) were dissolved in 250 ml. of absolute benzene, and a little of the benzene was distilled off in order to remove traces of moisture. Then 63 g. of freshly distilled ethyl bromoacetate and 56 g. of thoroughly dried, granulated zinc were added, the latter having been activated by washing in turn with warm NaOH solution, HCl, water (several times), alcohol and absolute ether. The mixture, contained in a flask fitted with a reflux condenser carrying a $CaCl_2$ tube, was gently warmed for about 15 min. until the reaction set in, and was then cooled until the reaction became less vigorous (about 40 min.); finally it was refluxed for a further 2 hr. It was then cooled, 300 ml. ice and water were added, followed by enough ice-cold 10 % H_2SO_4 to dissolve the precipitate. The solution was extracted thoroughly with ether (10 times) and the extract was dried over sodium sulphate and evaporated, leaving a colourless oil, which appeared to be insoluble in water. This ester was hydrolysed by boiling for 1½ hr. with a solution of 33 g. KOH in 20 ml. of water and 1400 ml. of ethyl alcohol. After adding 1 l. of water, the solution was evaporated to small bulk at a temperature not exceeding 50°, and the residue was extracted three times with ether, which removed any unchanged aldehyde and traces of unsaponified material. The aqueous solution and washings were then acidified with 50 % HCl, and extracted ten times with a large bulk of ether (about 5 l. in all). The solution was dried and the solvent was evaporated, leaving a residue which partially crystallized on standing, giving long needles, M.P. 70–75°.

The main bulk of the residue was distilled *in vacuo*, the fraction boiling below 120° (mainly bromoacetic acid) being discarded. The fraction boiling at 125–130°/13 mm. solidified at once in the receiver. It weighed 19 g., had M.P. 75–77°, and did not decolorize bromine water or $KMnO_4$ solution. A small amount of the distillate was purified by resublimation, M.P. 83–85°. Analytical data were exactly right for the free acid, or for the lactone hydrated with one molecule of water of crystallization, but could not fit either

the expected lactone or a dihydroxylactone with a somewhat similar formula, as shown below:

		C %	H %
Calc. for β-hydroxy-γγ-dimethyl-δ-valerolactone	$C_7H_{12}O_3$	58·3	8·4
,, α-β-dihydroxy-dimethyl-δ-valerolactone	$C_7H_{12}O_4$	52·5	7·56
,, Δα:β-γγ-dimethyl-δ-penteno-lactone	$C_7H_{10}O_3$	66·67	8·00
,, βδ-dihydroxy-γγ-dimethylvaleric acid	$C_7H_{14}O_4$	51·7	8·7
Found		51·53	8·58

An attempt to make the amide by the usual method of passing ammonia into the dry methyl alcoholic solution of the lactone and keeping for 48 hr., yielded, on addition of ether, glistening white platelets, M.P. 124–125°, which contained no N, and analysed approximately correctly for the hydrated lactone. This suggested that the lactone might be obtained in crystalline form from ether. About 3 g. of the lactone were taken up in absolute ether, the solution was evaporated slightly, and light petroleum was added until crystallization set in. The product was obtained in the form of needles (1·9 g.), M.P. 110–112°. On recrystallization from ether-light petroleum, it gave needles (1·1 g.), M.P. 126–127°, and a further recrystallization of these needles from a large bulk of light petroleum (B.P. 40–60°) gave glistening flat plates, M.P. 126–126·5°; the M.P. was unchanged by a further recrystallization. Either plates or needles could be obtained at will by seeding. (Found: C, 51·3; H, 8·59 %. Calc. for $C_7H_{14}O_4$: C, 51·7; H, 8·7 %.)

A Zerewitinoff determination on this material gave a result in excellent agreement with that calculated for the required lactone. (Active hydrogen found (in pyridine), 0·68 %; calc. for required lactone, 0·69 %; calc. for free acid, 1·23 %.)

Condensation. Freshly distilled lactone (535 mg.) dried over phosphorus pentoxide was dissolved in 4 ml. of freshly redistilled dry methyl alcohol and 416 mg. of β-alanine sodium salt were added. After refluxing for 1 hr., the mixture was worked up in the usual way. Yield, 700 mg. (Found: N, 4·92 %. Calc. for $C_{10}H_{18}O_5NNa$: N, 5·48 %.)

Dehydrohomopantothenic acid (15)

Δα:β-γγ-Dimethyl-δ-valerolactone. This was prepared, admixed with another lactone, the nature of which is uncertain and which is still under investigation, by the method described by Michael & Ross [1933]. A mixture of 42 g. of αα-dimethyl-β-hydroxypropaldehyde and 23 g. of malonic acid, 22 g. of acetic anhydride and 12 g. of glacial acetic acid, was refluxed for 40 hr. at about 140°. The solution was then distilled with a column up to 120° to remove acetic anhydride, and the residue was fractionated *in vacuo* at 25 mm. The distillate obtained below 130° was discarded, whilst the fraction boiling between 158 and 165° solidified in the receiver to a wax melting at 102–104°. In order to obtain the lactone pure, this crude product was treated as follows.

The crystalline mass was taken up in ether, and extracted with NaOH solution, and the aqueous solution was acidified with HCl, and ether-extracted. On evaporating the ether, the free acid which remained was lactonized by warming on the water bath for 15 min. with 10 % aqueous HCl. The solution was made alkaline with $NaHCO_3$ (in which the lactone was insoluble), and ether-extracted. The product (7 g.) crystallized in the form of needles, M.P. 102–106°, which, under the microscope, could be seen to be of two types, one clusters of fern-like needles, and the other long, thin needles. With homopantolactone it gave a depression of about 20°, and it decolorized $KMnO_4$ in the cold. On recrystallization from light petroleum, characteristic clusters of needles were obtained, M.P. 110–110·5°, which did not decolorize $KMnO_4$, whilst from the mother liquors, after five more recrystallizations, long needles were eventually obtained, which decolorized $KMnO_4$ in the cold, M.P. 115°, unchanged by two further recrystallizations. Analysis agreed fairly well with that for the required lactone. (Found: C, 67·28; H, 8·4 %. Calc. for $C_7H_{10}O_2$: C, 66·7; H, 8·00 %.)

Condensation. The unsaturated lactone (110 mg.) and 97 mg. of pure dry β-alanine Na salt were dissolved in 1 ml. of methyl alcohol and refluxed for 1½ hr. The mixture was worked up as usual. Yield, 160 mg. (Found: N, 7·30 %. Calc. for $C_{10}H_{16}O_4NNa$: N, 5·9 %. This may be taken to indicate *c.* 81 % condensation.)

bisnor*Deoxypantothenic acid* (*16*)

γ-Butyrolactone. This was prepared by the method described by Windaus [1921] and Fichter [1896] by reduction of succinic anhydride with sodium amalgam, B.P. 203–204°. The phenylhydrazide was prepared by refluxing a solution of 1·2 g. of butyrolactone and 1·62 ml. of redistilled phenylhydrazine in 25 ml. absolute alcohol with exclusion of moisture. After removal of the solvent *in vacuo*, the residue crystallized and was recrystallized from absolute alcohol-ether. M.P. 94–94·5°. Yield, 1·95 g. (Found: C, 61·90; H, 7·27; N, 14·7 %. Calc. for $C_{10}H_{14}O_2N_2$: C, 61·83; H, 7·21; N, 14·4 %.)

Condensation. 1 g. of freshly distilled lactone and 1·27 g. of β-alanine sodium salt were dissolved in 5 ml. of ethyl alcohol and treated as usual. Yield, 1·6 g. (Found: N, 7·9 %. Calc. for $C_7H_{12}O_4NNa$: N, 7·10 %. This may be taken to indicate *c.* 90 % condensation.)

isonor*Deoxypantothenic acid* (*17*)

γ-Valerolactone. This was prepared in the usual way by reduction of laevulinic acid [Fichter, 1903]. It was distilled at normal pressure immediately before use; B.P. 202–204° sharp. The phenylhydrazide was prepared as described under butyrolactone; 300 mg. gave 400 mg. of a derivative obtained crystalline after one week's standing in ether. M.P. 83–85°. (Found: C, 62·81; H, 7·60 %. Calc. for $C_{11}H_{16}O_2N_2$: C, 63·46; H, 7·69 %.)

Condensation. The lactone (510 mg.) and 566 mg. of β-alanine sodium salt were dissolved in 4 ml. of methyl alcohol and treated as usual. Yield, 700 mg. (Found: N, 7·71 %. Calc. for $C_8H_{14}O_4NNa$: N, 6·63 %. This may be taken to indicate 86 % condensation.)

The analogues with lysine, leucine, valine and taurine were prepared in the form of their calcium salts by a similar method. The calcium salts of the amino-acids were prepared by adding an excess of calcium hydroxide to the aqueous solution of the amino-acids, boiling for 1 min., evaporating to dryness under reduced pressure and removing the last traces of water by two more distillations after the addition of small amounts of absolute alcohol and absolute toluene. In this way, the calcium salt was obtained in the form of a dry white powder which was then condensed with α-hydroxy-ββ-dimethyl-γ-butyrolactone in the usual way.

Note. All melting points are uncorrected. Micro-analyses were carried out by Dr Weiler of Oxford. Our thanks are due to Miss P. Turner-Jones and Miss B. Wilson for assistance in preparing some of the intermediates in this work.

Discussion of results

It would already appear highly probable, from the results obtained by other workers on the activity of analogues of pantothenic acid (shown in Table 1) that pantothenic acid would prove to be highly specific as a growth stimulator, for the only analogue so far reported as having appreciable activity is hydroxypantothenic acid (no. 8, Table 1) which differs from pantothenic acid only in having a hydroxymethyl group instead of a methyl group on the β-carbon atom. However, the absence of one or both β-methyl groups appears to lead to complete (no. 7) or very considerable (nos. 5 and 6) loss of activity.

The present results tend to confirm this specificity of constitution. Deoxypantothenic acid (no. 13, Table 2) a compound identical with pantothenic acid except for the absence of the α-hydroxy group, is quite inactive. So also, as would be expected, are nos. 16 and 17 in which the α-OH and the two β-Me groups are absent. It is interesting to note here a point which will be dealt with in more detail in the following paper, namely, that loss of

the α-hydroxy group is not only associated with loss of growth-promoting activity, but also gives rise to the ability to inhibit bacterial growth. In fact, the three compounds, 13, 17 and 16 show increasing inhibitory activities in this order.

Homopantothenic acid, no. 14, differs from pantothenic acid only in having an additional CH_2 group in the straight chain of the lactone part of the molecule. It was therefore quite surprising to find that this compound was only 1/10,000 as active as pantothenic acid. Further, the unsaturated homologous compound was also inactive.

This result is in line with the observation of Weinstock [1940] that homologue no. 10 (Table 1) containing one more CH_2 than pantothenic acid, is inactive, although in this case of course the CH_2 is not in the straight chain.

The analogues with other amino-acids show no interesting replacement effects, whilst the remarkable inhibitory effects of pantoyltaurine (no. 21) will be dealt with in the next paper.

Other analogues are under investigation.

Summary

1. Various new analogues of pantothenic acid have been prepared by condensing β-alanine with five different lactones, and by condensing α-hydroxy-ββ-dimethylbutyrolactone with four different amino-acids.

2. In no instance was the analogue able to replace pantothenic acid as a growth-stimulator when tested on micro-organisms. Loss of the α-hydroxy group and increase in length of the carbon chain are associated with loss of biological activity.

3. Two new lactones have been prepared during the course of this work. They are β-hydroxy-γγ-dimethyl-δ-valerolactone and Δα:β-γγ-dimethyl-δ-valerolactone.

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40. Analogues of Pantothenic Acid

2. Preparation of Growth Inhibitors

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(*Received 25 March 1942*)

Of the various theories advanced to explain the bacteriostatic effect of sulphanilamide and related compounds, that which has received most widespread acceptance is the 'enzyme-interference' or the 'anti-metabolite' theory.

This theory is based on evidence published by Woods [1940] and Selbie [1940]. The former found that *p*-aminobenzoic acid antagonized the bacteriostatic effect of sulphanilamide *in vitro* and the latter obtained the same result *in vivo*. Fildes [1940] suggested that the bacteriostatic action of sulphanilamide was due to competition for an enzyme containing *p*-aminobenzoic acid, which he regarded as an essential metabolite for such organisms as are inhibited by sulphanilamide. The mutually antagonistic action of *p*-aminobenzoic acid and sulphanilamide was confirmed by Rubbo & Gillespie [1940], Spink & Jermsta [1941] and Strauss *et al.* [1941], whilst the general theory received support from McIlwain's observation that nicotinic acid and pyridine-β-sulphonic acid also antagonize one another [McIlwain, 1940; 1941]. (See Table 2 for formulae.)

Arising out of the work on pantothenic acid described in Part 1, it occurred to us that since pantothenic acid is known to be an essential metabolite for a number of organisms, it should be possible to prepare analogues of similar structure which would antagonize the effect of pantothenic acid, just as sulphanilamide neutralizes the growth-effect of *p*-aminobenzoic acid.

Table 1

No.	Amino-acid	Lactone	Analogue	Inhibitory effect
1	Taurine	α-Hydroxy-$\beta\beta$-dimethyl-γ-butyrolactone	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—SO_3H$ Pantoyltaurine	Highly inhibitory to *Strep. haemolyticus* and some strains of *Corynebact. diphtheriae*. Effect reversed by pantothenic acid
2	Taurine	β-Hydroxy-$\gamma\gamma$-dimethyl-δ-valerolactone	$CH_2OH—C(CH_3)_2—CHOH—CH_2—CO—NH—CH_2—CH_2—SO_3H$ Homopantoyltaurine	Inhibitory to *Strep. haemolyticus*, but less active than 1. Effect reversed by pantothenic acid
3	Taurine amide	As 1	$CH_2OH—C(CH_3)_2—CHOH—CO—NH—CH_2—CH_2—SO_2NH_2$ Pantoyltauramide	As 2
4	β-Alanine	$\beta\beta$-Dimethyl-γ-butyrolactone	$CH_2OH—C(CH_3)_2—CH_2—CO—NH—CH_2—CH_2—COOH$ Deoxypantothenic acid	Showed some inhibition to *B. coli*. Effect not reversed by pantothenic acid
5	β-Alanine	γ-Butyrolactone	$CH_2OH—CH_2—CH_2—CO—NH—CH_2—CH_2—COOH$ *bisnor*Deoxypantothenic acid	Inhibitory in high dilution towards *S. haemolyticus*, *C. diphtheriae*, *B. coli*, *Pr. vulgaris*, and *S. aureus*. With all organisms except *Pr. vulgaris*, effect not reversed by pantothenic acid
6	β-Alanine	γ-Valerolactone	$CH_2—CHOH—CH_2—CH_2—CO—NH—CH_2—CH_2—COOH$ *isonor*Deoxypantothenic acid	Similar to 5 but less marked

The preparation of pantoyltaurine (Table 1, no. 1) (for an explanation of the nomenclature adopted, see Part 1) was suggested to us by Dr H. McIlwain, and we accordingly prepared this compound and related substances which are listed in Table 1. While this work was in progress, two papers were published by Snell [1941, 1, 2] in which the preparation of pantoyltaurine (but not of any other analogues) was described.* Snell found that pantoyltaurine inhibited the growth of *Lactobacillus arabinosus*, *Streptococcus lactis* and *Propionibacterium pentosaceum*, but he did not apparently carry out any tests with *Streptococcus haemolyticus* or *Corynebacterium diphtheriae*, with which the most significant results in the present investigation were obtained. The present work was carried out independently of Snell, although we cannot claim priority in the preparation of pantoyltaurine.

The methods of testing these analogues for inhibitory effect on the growth of microorganisms and the results obtained will be discussed in detail in a subsequent communication by Dr H. McIlwain who performed the bacteriological tests. The compounds were tested on *Bact. coli*, *Proteus morgani*, *Pr. vulgaris*, *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Corynebacterium diphtheriae*; the results are summarized in Table 1.

Toxicity tests indicate that pantoyltaurine is non-toxic to mice in a dose of 5 g. per kg. *In vivo* animal tests are in progress, and these results will be published in a later communication.

Chemical method

The general method of condensation was that described in Part 1, where the preparation of compounds 4, 5 and 6 has already been given, and the preparation of the taurine analogues will be described here. With compounds 1 and 2, the method of refluxing the components together in methyl alcohol was found more convenient than the fusion method [Williams, 1940; Snell, 1941, 1, 2], the latter yielding a solid glass which was difficult to handle. Both products, however, showed a similar degree of inhibition. With pantoyltauramide, which was soluble in ether, the refluxing method had no advantage over the fusion method.

Taurine sodium salt

Taurine, M.P. 299° with decomposition, was prepared from ox-bile. The sodium salt was prepared by adding an exact equivalent of 10% NaOH, boiling for 1 min., filtering from a trace of flocculent precipitate which always appeared, and evaporating to dryness *in vacuo*. Addition of absolute alcohol and toluene, followed by evaporation to dryness was carried out three times, to ensure that the product was quite dry. It was finally ground up with ethyl alcohol, filtered, and washed with absolute alcohol and absolute ether. (Found: N, 9·3%. Calc.: N, 9·53%.)

Condensation

Pantoyltaurine (*no.* 1). The condensation of pantolactone with taurine was effected both by the fusion method and by the refluxing method.

(*a*) *Fusion method.* The sodium salt of taurine (581 mg.) and pure dry pantolactone (502 mg.) were heated together in an oil-bath at 120° for 3 hr. Yield 600 mg. (Found: N, 4·81%. Calc.: N, 5·06%.)

(*b*) *Refluxing method.* The same amounts of the reactants, as above, were refluxed in 5 ml. of absolute methyl alcohol for 3½ hr. (Refluxing for 1 hr. only, gave incomplete condensation.) Yield 750 mg. (Found: N, 5·10%. Calc.: N, 5·06%.)

Homopantoyltaurine (*no.* 2). The sodium salt of taurine (581 mg.) was fused with 550 mg. of homopantoyllactone (β-hydroxy-γγ-dimethyl-δ-valerolactone) at 120° for 3 hr. in the usual way. Yield 700 mg. (Found: N, 4·38%. Calc.: N, 4·81%.)

* There has recently come to our notice a paper by Kuhn *et al.* [1941] in which the preparation of the *d*- and *l*-pantoyltaurines is described, and their inhibitory effects on the growth of *Streptobacterium plantarum* noted.

Pantoyltauramide (*no.* 3). Taurine amide hydrochloride was prepared by the method of Miller [1940], M.P. 132–133°. The free amide was obtained from the hydrochloride by dissolving the latter in water, adding an exact equivalent of 20% aqueous NaOH and evaporating the water *in vacuo* at 50°. Absolute ethyl alcohol and toluene were added and distilled off *in vacuo* at 50°, this procedure being repeated twice more to remove all traces of water. Finally, five parts of absolute ethyl alcohol were added, the precipitated sodium chloride was filtered off and washed well with alcohol, and the filtrate was evaporated to dryness *in vacuo* at 40°. The residue crystallized at once in flat platelets.

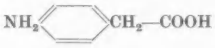
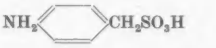
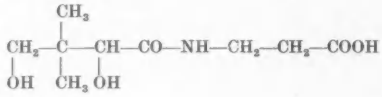
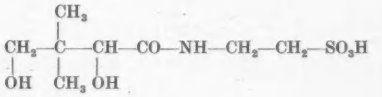
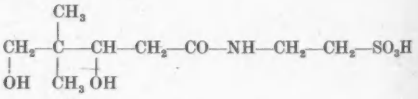
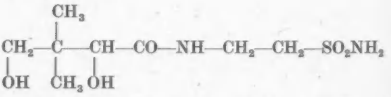
Condensation. Taurine amide (1·11 g.) and pantolactone (1·17 g.) were heated together for $3\frac{1}{2}$ hr. at 120°. The mass melted to a viscous liquid, but did not give a precipitate when a trial portion was dissolved in methyl alcohol and added to ether, and it was therefore tested in the form of a yellowish glass-like solid.

Note. All melting points are uncorrected. Micro-analyses were carried out by Dr G. Weiler of Oxford. Our thanks are due to Misses E. Paxton, K. N. Speyer, P. Turner-Jones and B. Wilson for assistance in the preparation of some of the intermediate compounds, and to Mr A. L. Bacharach for the toxicity determination.

Discussion

The bacteriological results with analogues 1, 2 and 3 provide further confirmation of the general anti-metabolite theory, of which three examples have already been reported in the literature. These are given, together with the substances now described, in Table 2;

Table 2. *Table showing pairs of substances so far found to be mutually antagonistic to one another*

No.	Growth stimulator	Corresponding inhibitor
1		
2		
3		
4		
5	As 4	
6	As 4	

References: No. 1, Fildes [1940], Woods [1940]; No. 2, McIlwain [1940]; No. 3, Rubbo & Gillespie [1942]; No. 4, Snell [1941, 1, 2]; Kuhn *et al.* [1941].

the components of each pair of compounds are mutually antagonistic; that is, the effect of one may be nullified by addition of a sufficient quantity of the other. In each instance, the two compounds are closely related chemically. The degree of inhibition by pantoyltaurine depends on the amount of pantothenic acid present in the medium and is reversed in a regular manner by addition of pantothenic acid, just as in the case of *p*-aminobenzoic

acid and sulphanilamide. It might be worth noting here that the chemical structure of the inhibitor does not seem to be necessarily so specific as that of the growth-promoter, since homopantoyltaurine (Table 2, no. 5) inhibits growth and is reversed by pantothenic acid, whereas its analogue homopantothenic acid (Part 1) does not act as a growth-stimulator.

The inhibition shown by compounds 4, 5 and 6 (Table 1), all of which lack the α-hydroxy group of pantothenic acid, appears to be of a different type from that produced by the sulphonic acids, for the inhibition is not reversed by addition of pantothenic acid. These three analogues (nos. 4, 6 and 5, Table 1) show an increasing degree of inhibition in that order and this may be correlated with an increasing divergence in structure from that of pantothenic acid. The inhibition produced by the sulphonic acids is of the competitive type, whereas the inhibition produced by the deoxypantothenic acids is of the non-competitive type, since it is not reversed on addition of pantothenic acid.

The preparation of further analogues is in progress.

Summary

1. Analogues of pantothenic acid obtained by condensing α-hydroxy-ββ-dimethylbutyrolactone with taurine and taurine amide and by condensing β-hydroxy-γγ-dimethylvalerolactone with taurine have been prepared and have been found to be highly inhibitory to *Streptococcus haemolyticus* and to some strains of *Corynebacterium diphtheriae*. The inhibitory effect is reversed by pantothenic acid.

2. Other analogues have been prepared which show an inhibitory activity which is not reversed by pantothenic acid. In all these compounds, the α-hydroxy group of pantothenic acid is absent.

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41. The Preparation of 3:5-Dinitrobenzoic Acid and 3:5-Dinitrobenzoyl Chloride

Observations on the Acylation of Amino-acids by means of 3:5-Dinitrobenzoyl Chloride and certain other Acid Chlorides

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(*Received 9 March 1942*)

3:5-Dinitrobenzoyl chloride was suggested by one of us [Saunders, 1934; 1938] as a useful reagent for the identification of certain classes of amino-acids. The preparation of this reagent has now been examined in detail. The two recorded preparations of 3:5-dinitrobenzoic acid from benzoic acid [Shukow, 1895; Sirks, 1908] are long, the former author recommending the heating of benzoic acid with fuming nitric acid and concentrated sulphuric acid for 8–10 hr. on a water bath and then for 1–2 hr. on a sand bath. The second author heated the mixture on a water bath for 12 hr., poured into water and then repeated the nitration on the product for a further 12 hr. Details are given below for the preparation of 3:5-dinitrobenzoic acid in which, by careful temperature control, the period of nitration for a corresponding weight of benzoic acid has been reduced to $2\frac{1}{2}$ hr. Moreover, Sirks's yield has been improved upon and the quantities of nitrating acid used by Shukow have been reduced by 15%.

The recorded preparation of 3:5-dinitrobenzoyl chloride [Berend & Heymann, 1904] consists of heating the acid with phosphorus pentachloride and ultimately distilling the product under reduced pressure at the rather high temperature of 200°. During this process, the 3:5-dinitrobenzoyl chloride is liable to explode. Furthermore, the separation of the solid acid chloride in the condenser may be very troublesome. A more convenient method is now described in which the final distillation is avoided. The pure product has M.P. 69·5° which is close to that of 68–69° given by Eastman Kodak [1934]. Cohen & Armes [1906] give 66–68°. The higher M.P. of 74° obtained by Berend & Heymann [1904] and by Town [1941] has not been observed by us, even after distillation at 172° and 15 mm.

The method of 3:5-dinitrobenzoylation used in the above communications [Saunders, 1934; 1938] was rapid and simple and consisted of dissolving the amino-acid in *N* NaOH and shaking the solution for about 2 min. with finely powdered 3:5-dinitrobenzoyl chloride. The latter dissolved extremely rapidly, and on acidification the 3:5-dinitrobenzoyl derivative was precipitated.

It was found that neutral and basic ampholytes gave crystalline derivatives in good yield, whereas acidic ampholytes were usually less reactive, and, under the conditions of the experiment, glycine could be separated, for example, from aspartic acid. Tyrosine gave no derivative.

It was also shown that the reagent could be used for separating certain 'reactive' amino-acids, for example, leucine and glycine. On adding dilute acetic acid to an alkaline solution of the two derivatives, 3:5-dinitrobenzoylleucine was precipitated while 3:5-dinitrobenzoylglycine, which remained in solution, could be precipitated by dilute HCl.

The principle of fractional precipitation at different *p*H has recently been extended by Town [1941] to the separation of the isomers of valine and leucine. In this paper, Town claims that, in the presence of NaOH, 'reaction times for the benzoylation of the order of 30 min. have been found'. This is contrary to our experience, which has shown that one

great advantage of this reagent as compared with most other similar reagents, is that the reaction is extremely rapid and is usually complete in less than 2 min.

It is difficult to see how, in the presence of NaOH, the 'reaction time' can be greater than 2 min., for we have shown that 1 mol. of 3:5-dinitrobenzoyl chloride, in the absence of amino-acid, is almost completely dissolved and hydrolysed by 2 mol. NaOH in 2 min. Hence, if in the 3:5-dinitrobenzoylation of an amino-acid, the latter does not react within 2 min., the acid chloride uses up the alkali by the process of rapid hydrolysis, and no further reaction can take place.

It should be pointed out that our technique differs from that of Town in that we add the powdered acid chloride in one lot to the alkaline solution of the amino-acid. Town, on the other hand, adds the acid chloride in small quantities at a time, but this in itself cannot cause the actual 'reaction time' to be longer.

Our experiments show that with a solution of glycine in NaOH, no advantage is gained by adding the acid chloride in small quantities at a time over 30 min.

One mol. of acid chloride requires 2 mol. NaOH for hydrolysis, and so it seemed of nterest to compare the amounts of certain acid chlorides (used in amino-acid identification) left after 2 min. when acted upon by a slight excess of *N* NaOH. From the results given in the Experimental Section, it will be seen that among the aromatic acid chlorides, 3:5-dinitrobenzoyl chloride and *m*- and *p*-nitrobenzoyl chlorides are in a class by themselves, the hydrolysis being complete within this period. Of course, it is not to be assumed that the reaction of a particular acid chloride with the OH^- ion will exactly correspond to that with an $—NH_2$ group, as far as order of reactivity is concerned.

The reaction between 3:5-dinitrobenzoyl chloride and aniline in ethyl acetate solution, and also that between benzoyl chloride and aniline, in the same solvent, have been investigated. Ethyl acetate was chosen as solvent because of the high solubility of the anilides produced and the almost complete insolubility of aniline hydrochloride in this liquid. The yields of aniline hydrochloride which had separated after 2 min with 3:5-dinitrobenzoyl chloride and benzoyl chloride were 95 and 78 % of the possible amount, respectively. Under similar conditions, using *p*-toluenesulphonyl chloride, no aniline hydrochloride had separated after 2 min. Even with methanesulphonyl chloride (which is very reactive toward OH^- ions) no precipitate appeared within 2 min. It is proposed, at a later stage, to investigate this phenomenon in greater detail.

The 3:5-dinitrobenzoylation of aniline was also examined. Aniline was chosen because of the ready separation of 3:5-dinitrobenzanilide, in a reasonably pure condition, from the reaction mixture, which contained varying quantities of aqueous NaOH. It was again shown that no advantage was to be gained by adding the acid chloride in small quantities over 30 min.

It was pointed out in our previous communications that very little 3:5-dinitrobenzoic acid was formed during the acylation of the more reactive amino-acids. On the other hand, with the less reactive amino-acids, considerable quantities of 3:5-dinitrobenzoic acid were sometimes produced. In support of this, the yields of 3:5-dinitrobenzoic acid obtained, were frequently quoted, and the fact that derivatives precipitated by dilute acetic acid were free from 3:5-dinitrobenzoic acid, was stressed.

In looking for a possible alternative rapid reagent for the identification of amino-acids and one which would give a product free from organic acid on acidifying with HCl, it seemed that 3:5-dinitrobenzenesulphonyl chloride might meet the case, as the corresponding acid is very soluble in water. Its reactivity, however, was found to be of a low order.

Methanesulphonyl chloride was found to be very reactive towards OH^- ions. The possibility of using this reagent for identifying amino-acids was first investigated by Helferich & Mittag [1938], but a quicker method for obtaining the glycine derivative (although in smaller yield) than that described by these authors, is given below. However,

one drawback to the general use of this reagent in the case of amino-acids, is the high solubility of the methanesulphonyl derivatives in water. Furthermore, from work so far done, it appears that methanesulphonyl chloride is less reactive towards the NH_2 group than towards OH^- ions. It seems that methanesulphonyl chloride is a suitable and very rapid reagent for the identification of certain phenols. It does not appear to react to any extent with salicylic acid or with tyrosine, in which respect it resembles 3:5-dinitrobenzoyl chloride.* In this connexion, it should be noticed that with substances (e.g. phenol, aniline) which give 3:5-dinitrobenzoyl derivatives insoluble in NaOH, no reaction of any appreciable extent takes place if the reactants are shaken together in a stoppered bottle in the usual way. The solid acid chloride becomes coated immediately with a thin layer of the derivative, thus preventing any further reaction. The reaction can be caused to take place if the reactants are thoroughly ground together in a mortar.

The higher aliphatic sulphonyl chlorides, such as ethane-, *n*-propane- and *n*-butane-sulphonyl chlorides, give derivatives of phenols of lower M.P. and, moreover, the *n*-butane-sulphonyl chloride shows a marked decrease in reactivity.

α-Toluenesulphonyl chloride is slightly less reactive than methanesulphonyl chloride, but the reaction with glycine is complicated by the simultaneous production of sodium α-toluenesulphonate.

Town's paper [1941] is also concerned with the isolation from a specimen of *dl*-valine of a substance which he believed to be 3:5-dinitrobenzoyl*nor*valine, and from a specimen of leucine, of a substance which he suggested might be 3:5-dinitrobenzoyl*nor*leucine, the identity of both these compounds being subject to confirmation. We have prepared the 3:5-dinitrobenzoyl derivatives of *dl-nor*valine (*dl*-α-amino-*n*-valeric acid) and of *dl-nor*leucine (*dl*-α-amino-*n*-hexoic acid) and we find that their melting points do not correspond with those recorded by Town. In this connexion, the possibility of dimorphism among dinitrobenzoyl derivatives must not be overlooked, particularly as dimorphism among other dinitro compounds is known, as, for example, the dimorphism of the 2:4-dinitrophenylhydrazone of acetaldehyde [Bryant, 1933; Martin *et al.* 1941].

The melting points of a few of the derivatives obtained by one of us [Saunders, 1934; 1938] are different from those recorded in the literature [Town, 1941]. 3:5-Dinitrobenzoyl derivatives have a marked tendency to form hydrates, and these differences of melting point may be due to the separation of different hydrates.

It should also be noted that the melting points of certain hydrates depend upon the rate of heating, as already reported, e.g. for 3:5-dinitrobenzoylsarcosine [Saunders, 1934].

Experimental

3:5-Dinitrobenzoic acid

Benzoic acid (50 g.) was dissolved in conc. H_2SO_4 (230 ml.) in a litre flask which was then fitted with a ground-in condenser. Nitric acid (73 ml., sp.gr. 1·5) was then added, a few ml. at a time, the flask being well shaken and cooled in ice-water during the addition. Much heat was evolved and a clear yellow solution was obtained. After adding porous pot, the mixture was heated gradually in a water bath up to 100° during 45 min. At 70–80° the reaction tended to become vigorous and was moderated, when necessary, by immersing the flask in cold water. The mixture was maintained at 100° for 15 min., with occasional shaking, and then transferred to an oil bath at 100° and the temperature raised to 130° over 30 min. and then kept between 130 and 140° for 1 hr. (The total period of heating was thus 2½ hr.) The flask was allowed to cool and at about 90°, crystals began to separate, the process of crystallization being accelerated by shaking. When cold, the

* Unlike salicylic acid, methyl salicylate is readily dinitrobenzoylated. It has also now been shown that 3:5-dinitrobenzoyl chloride does not react with mandelic acid in the presence of NaOH to any appreciable extent.

contents of the flask were poured into 3–4 l. of ice-water and the crystals filtered off. After washing with water and drying they had M.P. 204° and were sufficiently pure, without recrystallization, for most purposes. (Found: C, 39·6; H, 2·04. Calc. for $C_7H_4O_6N_2$: C, 39·6; H, 1·90 %.) After recrystallization from water, the acid was obtained in characteristic rhombs. M.P. 204°.

3:5-Dinitrobenzoyl chloride

3:5-Dinitrobenzoic acid (30 g., 1 equiv.) was mixed with PCl_5 (33 g., 1·1 equiv.) and the mixture was heated under reflux in an oil bath at 120–130° for 1¼ hr. The phosphorus oxychloride was then distilled off at 25° (20 mm.), the temperature of the bath being finally raised to 110° during this process. On cooling, the residue in the flask solidified to a brown mass of crude 3:5-dinitrobenzoyl chloride (yield, theoretical; M.P. 63° to a clear liquid). Carbon tetrachloride proved to be a very suitable solvent for recrystallization, particularly for large quantities. Yield of recrystallized material, 25 g. (77 %), M.P. 66–68°. Beautiful long white needles of pure material, with M.P. 69·5°, were obtained by recrystallization from light petroleum (B.P. 40–60°), but a relatively large amount of solvent was required. (Found: N, 12·2; Cl, 15·5. Calc. for $C_7H_3O_5N_2Cl$: N, 12·2; Cl, 15·4 %.)

3:5-Dinitrobenzoyl dl-α-*amino*-n-*valeric acid*

The amino-acid (1·17 g., 0·01 mol.) was dissolved in *N* NaOH (20 ml., 0·02 mol.) and finely powdered 3:5-dinitrobenzoyl chloride (2·31 g., 0·01 mol.) added. The mixture was shaken in a stoppered bottle for 2 min. Practically the whole of the solid dissolved very rapidly, and after filtration, the solution was acidified with dilute acetic acid. The white precipitate (2·12 g.) thus obtained was twice recrystallized from aqueous methyl alcohol. M.P. 227·5–228·5° to a brownish liquid. (Found: C, 46·5; H, 4·38. $C_{12}H_{13}O_7N_3$ requires: C, 46·3; H, 4·22 %.)

3:5-Dinitrobenzoyl dl-α-*amino*-n-*hexoic acid*

The derivative was prepared similarly to that of *dl*-α-amino-*n*-valeric acid. Yield of crude compound, 77·3 %. Recrystallized twice from aqueous ethyl alcohol. M.P. 203·5–204°. (Found: C, 47·9; H, 4·92. $C_{13}H_{15}O_7N_3$ requires: C, 48·0; H, 4·65 %.)

3:5-Dinitrobenzoyl dl-α-*methyl*-α-*amino*-n-*butyric acid*

Prepared from *dl*-α-methyl-α-amino-*n*-butyric acid (0·88 g., 0·0075 mol.) dissolved in *N* NaOH solution (15 ml.) and mixed with 3:5-dinitrobenzoyl chloride (1·73 g., 0·0075 mol.). On acidifying the alkaline solution with dilute acetic acid, a white precipitate (A, 0·91 g.) was obtained. On further acidification of the filtrate from A with dilute hydrochloric acid, 3:5-dinitrobenzoic acid was obtained, the yield being 0·7 g. Two recrystallizations of A from aqueous methyl alcohol gave white flakes. M.P. 186°. (Found: C, 46·4; H, 4·53. $C_{12}H_{13}O_7N_3$ requires: C, 46·3; H, 4·22 %.)

3:5-Dinitrobenzoylglycine

(i) Prepared according to our previous method [1934] by shaking glycine (0·38 g.) in *N* NaOH solution (10 ml.) with 3:5-dinitrobenzoyl chloride (1·16 g.) for 2 min. Very little acid chloride remained to be filtered off. Yield of product on acidification: 1·17 g. (86 %). M.P. of crude material: 169–175°.

We originally gave the melting point of the recrystallized material as 179° which is close to Adamson's [1939] figure of 178°. Town gives M.P. 182·4°. We now find that even after three recrystallizations (10 % acetic acid, water, water plus animal charcoal) the melting point is substantially the same at 179·5°.

(ii) Prepared as above but the acid chloride was added in small quantities at a time over 30 min. Yield of product: 1·14 g. (84 %). M.P. 168–175°.

Reaction between various acyl chlorides and N *sodium hydroxide solution*

To a weighed quantity of the acyl chloride, a measured volume of *N* NaOH solution was added and the mixture shaken for 2 min. The unchanged chloride (if solid) was then immediately filtered off, washed, dried and weighed.

The solids were recrystallized to constant melting point before the experiment and the melting point of the unreacted material was taken in each case to ensure that it had undergone no essential change. The solid chlorides were finely powdered before use. In the experiments with liquid chlorides, the unchanged substances were extracted after 2 min. by quickly pouring the mixture into 25 ml. of ether in a separating funnel and shaking. After separating and drying over calcium chloride, the ether was distilled off from a previously weighed flask which was then gently reheated and allowed to cool in a dry atmosphere until a constant weight was obtained.

The results, which are, of course, only roughly quantitative, are given in the accompanying table.

Acid chloride used	Amount of chloride used g.	Amount of *N* NaOH ml.	Amount of chloride left after 2 min. g.	Percentage which had reacted
Methanesulphonyl	1·15	25	Disappeared almost immediately	100
Ethanesulphonyl	1·29	25	Disappeared almost immediately	100
n-Propanesulphonyl	1·43	25	Disappeared within 30 sec.	100
α-Toluenesulphonyl	0·95	12·5	0·06	94·2
β-Naphthalenesulphonyl	1·13	12·5	1·04	8·0
p-Toluenesulphonyl	0·95	12·5	0·88	7·4
3:5-Dinitrobenzenesulphonyl	0·67	6·25	0·58	13·4
Benzoyl	1·41	25	0·44	68·8
o-Nitrobenzoyl	1·86	25	0·21	88·7
m-Nitrobenzoyl	0·93	12·5	0·00 Disappeared within 90 sec.	100
p-Nitrobenzoyl	0·93	12·5	Disappeared within 90 sec.	100
3:5-Dinitrobenzoyl	1·16	12·5	Disappeared in about 40 sec.	100
3:5-Dinitrobenzoyl	1·16	10	0·01	99·1

Reaction between (i) *3:5-dinitrobenzoyl chloride and aniline in ethyl acetate solution,* (ii) *benzoyl chloride and aniline in ethyl acetate solution*

The reaction considered was: $2C_6H_5NH_2 + RCOCl = C_6H_5NH_2.HCl + RCONHC_6H_5$.

Aniline (0·93 g., 0·01 mol.) and 3:5-dinitrobenzoyl chloride (1·15 g., 0·005 mol.) were each dissolved in separate quantities of 40 ml. of ethyl acetate. On mixing the solutions, a white precipitate of aniline hydrochloride came down immediately. After 2 min., this was filtered off as rapidly as possible and, after drying, had M.P. 197–198° (M.P. of aniline hydrochloride, 198°). Light petroleum (B.P. 60–80°) added to the filtrate produced a white crystalline precipitate of M.P. 234° (3:5-dinitrobenzanilide has M.P. 234°).

An exactly parallel experiment to the above, but using the correct molar quantity of freshly distilled benzoyl chloride (B.P. 197·5–198°) in place of the 3:5-dinitrobenzoyl chloride, was carried out.

The results obtained in each case are given below:

Amount of aniline in 40 ml. of ethyl acetate g.	Amount of 3:5-dinitrobenzoyl chloride in 40 ml. of ethyl acetate g.	Amount of benzoyl chloride in 40 ml. of ethyl acetate g.	Yield of aniline HCl %	M.P. of anilide
0·93	1·15	—	95	234°
0·93	—	0·70	80	162°

Reaction between aniline and 3:5-dinitrobenzoyl chloride

In Exp. I, aniline, 3:5-dinitrobenzoyl chloride and *N* NaOH were ground together in a mortar for 2 min. and the precipitated 3:5-dinitrobenzanilide was filtered off, washed well, and again ground with 20 ml. of *N* NaOH to remove any unchanged acid chloride,

filtered off, and the two filtrates combined. This solution was concentrated and acidified with dilute HCl to precipitate the 3:5-dinitrobenzoic acid formed by hydrolysis of the acid chloride. In Exps. II and III, the NaOH was in excess and so it was not necessary to grind up the precipitated 3:5-dinitrobenzanilide with an extra amount of NaOH in order to remove unchanged acid chloride. Exp. IV resembled Exp. I except that the acid chloride was added in small quantities at a time over 30 min.

Exp.	Amount of acid chloride g.	Amount of *N* NaOH ml.	Yield of 3:5-dinitrobenzanilide %	Yield of 3:5-dinitrobenzoic acid %
I	0·93	10	72	21
II	0·93	20	70	20
III	0·93	30	66	24
IV	0·93	10	68	23

3:5-Dinitrobenzoyl methyl salicylate

Methyl salicylate (1·52 g., 0·01 mol.), 3:5-dinitrobenzoyl chloride (2·31 g.) and *N* NaOH (10 ml.) were ground together in a small mortar for 2 min. The reaction mixture, an orange liquid and a paste-like mass, was treated with ether and the white solid so obtained was filtered off and twice recrystallized from a mixture of chloroform and light petroleum (B.P. 40–60°). It formed small, colourless needles; M.P. 107·5°. (Found: C, 52·0; H, 3·04. $C_{15}H_{10}O_8N_2$ requires C, 52·0; H, 2·91 %.)

Reaction between 3:5-dinitrobenzoyl chloride and dl-*mandelic acid*

dl-Mandelic acid (1·52 g., 0·01 mol.) was dissolved in *N* NaOH (30 ml., 0·03 mol.) and powdered 3:5-dinitrobenzoyl chloride (2·31 g., 0·01 mol.) was added and the mixture shaken for 2 min. A small quantity (0·13 g.) of unchanged acid chloride was filtered off and the filtrate acidified with dilute HCl. After cooling and standing for some time, a pale yellow precipitate, A, separated. The filtrate from A was evaporated to dryness and the solid so obtained extracted with boiling acetone. The part (NaCl, 1·65 g.) insoluble in acetone was filtered off and the solution was evaporated to dryness, producing a slightly sticky solid which was recrystallized from benzene in white flakes (B).

Substance A (1·91 g.) had M.P. 204–205°, and proved to be 3:5-dinitrobenzoic acid. Yield: 95·7 %, allowing for the small quantity of acid chloride recovered unchanged. Substance B (1·33 g.) had M.P. 104–114°, and when once recrystallized from benzene, melted at 118–119° (*dl*-mandelic acid has M.P. 118°). Yield 87·5 %.

m-*Nitrobenzoylglycine*

This substance was prepared by direct *m*-nitrobenzoylation, a method which does not seem to have been used previously. It had been prepared from benzoylglycine by nitration [Conrad, 1877], and also from aminoacetonitrile and *m*-nitrobenzoyl chloride, followed by hydrolysis [Klages & Haack, 1903].

Glycine (0·75 g.) was dissolved in *N* NaOH (20 ml.) and powdered *m*-nitrobenzoyl chloride (1·86 g., 0·01 mol.) added. The whole was shaken for 2 min., after which practically all the acid chloride had dissolved. The filtered liquid gave no precipitate with acetic acid, but on the addition of dilute HCl, an emulsion formed and soon deposited white crystals (1·86 g.). These had M.P. 161–163°. After two recrystallizations from water, white needles were obtained having M.P. 166°. Melting points given in the literature are: 162° [Conrad], 165° [Klages & Haack], 165–167° [Sieber & Smirnow, 1887].

3:5-Dinitrobenzenesulphonyl glycine

The 3:5-dinitrobenzenesulphonic acid was prepared by the method of Griffith [1924], and converted into the sulphonyl chloride by the method of Jackson & Earle [1903].

Glycine (0·75 g., 0·01 mol.) was dissolved in *N* NaOH (30 ml., 0·03 mol.) and 3:5-dinitrobenzenesulphonyl chloride (2·67 g., 0·01 mol.) was added, the mixture being well shaken for 1½ hr. The 3:5-dinitrobenzenesulphonyl chloride (0·06 g., M.P. 103°) which had not dissolved, was filtered off and the filtrate acidified with dilute HCl, being then evaporated on the water bath until crystallization commenced. The brownish crystals which separated were extracted with ether, leaving a residue (2·4 g.). This residue was recrystallized, in small yellow flakes, from a relatively large volume of alcohol. (Found: N, 10·1. Sodium 3:5-dinitrobenzenesulphonate, $C_6H_3O_7N_2SNa$, requires: N, 10·4%.)

The ethereal extract, on evaporation to dryness, gave a substance (0·25 g.) which was recrystallized once from ether and twice from water to give almost colourless flakes with M.P. 191–192°. (Found: C, 32·0; H, 2·38; N, 14·1. The glycine derivative, $C_8H_7O_8N_3S$, requires: C, 31·5; H, 2·32; N, 13·8%.)

Methanesulphonyl glycine

The methanesulphonyl chloride was prepared from methyl thiocyanate [Johnson & Douglass, 1939].

Glycine (0·75 g., 0·01 mol.) was dissolved in *N* NaOH solution (20 ml., 0·02 mol.) and methanesulphonyl chloride (1·15 g., 0·01 mol.) added, and the whole shaken for 2 min. A clear, colourless solution was produced and acidified with dilute HCl, but as no precipitate appeared, even on standing, the liquid was evaporated on the water bath until crystals formed on cooling. After drying, they were extracted with boiling alcohol to separate any sodium chloride which they might have contained. From the filtered extract, white platelets separated and were halogen-free. After a second recrystallization from alcohol, the compound had M.P. 172–173°. (Found: C, 23·8; H, 4·75. Calc. for $C_3H_7O_4NS$: C, 23·5; H, 4·61%.)

Methanesulphonyl anthranilic acid

Prepared similarly to the glycine derivative. After 2 min. shaking, the clear solution was acidified with dilute HCl, when a whitish, granular precipitate separated. It was recrystallized from water (charcoal) in radiating tufts of white needles: M.P. 190·5–191·5°. (Found: C, 44·5; H, 4·40. $C_8H_9O_4NS$ requires: C, 44·6; H, 4·22%.)

Phenyl methanesulphonate

Phenol (1·88 g., 0·02 mol.) was dissolved in *N* NaOH (30 ml., 0·03 mol.) and methanesulphonyl chloride (2·29 g., 0·02 mol.) was added. A turbidity was immediately produced and a white crystalline precipitate (2·01 g.) separated during 2 min. shaking. In the process of recrystallization from water, the substance tended to appear first as an oil which gave small white flaky crystals on further cooling: M.P. 59·5°. (Found: C, 48·9; H, 5·10. $C_7H_8O_3S$ requires: C, 48·8; H, 4·68%.)

p-*Nitrophenyl methanesulphonate*

Prepared similarly to the phenol derivative, from *p*-nitrophenol (1·39 g., 0·01 mol.) suspended in *N* NaOH (15 ml., 0·015 mol.), and methanesulphonyl chloride (1·15 g., 0·015 mol.). The crude product was washed well with water and recrystallized from 90% ethyl alcohol in practically colourless crystals: M.P. 93–93·5°. They contained N and S, and gave no coloration with ferric chloride solution. (Found: C, 38·7; H, 3·32. $C_7H_7O_5NS$ requires: C, 38·8; H, 3·25%.)

α-Toluenesulphonyl glycine

The sulphonyl chloride was prepared according to the method of Johnson & Ambler [1914].

Glycine (0·75 g.) was dissolved in *N* NaOH (30 ml., 0·03 mol.) and shaken with α-toluenesulphonyl chloride (1·91 g., 0·01 mol.). After shaking for 15 min., the solution

was filtered from a small quantity of unchanged chloride, acidified with dilute HCl and evaporated until crystals separated. These were extracted with boiling chloroform. The undissolved portion (1·1 g.) was recrystallized from methyl alcohol as white flaky crystals. They proved to be sodium α-toluenesulphonate. (Found: C, 43·1; H, 3·85. $C_7H_7O_3SNa$ requires: C, 43·3; H, 3·64 %.)

The chloroform solution deposited crystals (0·18 g.) on concentration. These were twice recrystallized from chloroform and had M.P. 152°. The substance was α-toluenesulphonylglycine. (Found: C, 47·3; H, 5·09. $C_9H_{11}O_4NS$ requires: C, 47·2; H, 4·84 %.)

Summary

1. An improved method for preparing 3:5-dinitrobenzoic acid, involving a great reduction in the time of nitration, is described.

2. The preparation of 3:5-dinitrobenzoyl chloride is described.

3. Our previous observation [Saunders, 1934; 1938] that the 3:5-dinitrobenzoylation of amino-acids in normal sodium hydroxide solution is complete in 2 min., has been confirmed. 3:5-Dinitrobenzoyl chloride itself is completely dissolved and hydrolysed by a slight excess of normal sodium hydroxide solution within 2 min.

4. The 3:5-dinitrobenzoyl derivatives of *dl*-α-amino-*n*-valeric, *dl*-α-amino-*n*-hexoic and *dl*-α-methyl-α-amino-*n*-butyric acids are described. The two first-named derivatives do not possess the melting points of the compounds isolated by Town [1941] from specimens of valine and leucine, and described by him as probably being 3:5-dinitrobenzoyl*nor*valine and 3:5-dinitrobenzoyl*nor*leucine respectively.

5. There is evidence to show that the 3:5-dinitrobenzoyl derivatives of certain amino-acids can exist in different forms, particularly where hydrates are concerned.

6. The relative merits of 3:5-dinitrobenzenesulphonyl chloride, α-toluenesulphonyl chloride, methanesulphonyl chloride and other acid chlorides as acylating agents for amino-acids are discussed.

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42. The Detection of Insulin in Urine

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(*Received 18 March 1942*)

The presence or absence of insulin in the urine of either normal or diabetic persons is still a matter of dispute, for the findings of the various investigators are conflicting. Best & Scott [1923] claimed that insulin was eliminated by the kidneys under certain conditions. They found that the equivalent of 800 ml. of normal urine, when injected into a depancreatized dog, depressed the blood sugar considerably over a long period, but they were unable to recover any insulin from the urine of diabetic patients unless they were having insulin treatment. Partos [1929] cited evidence that insulin was a normal component of both human and rabbit urines though he could not detect it in urine from diabetic patients. Weiler *et al.* [1934] have confirmed these findings and Kozuka [1927] isolated a blood sugar-depressing substance (which he believed to be insulin) from normal urine but could not extract it from diabetic urine unless the patient was receiving insulin and not excreting sugar. Brugsch & Horsters [1930] found an insulin-like substance in the urine of both normal and diabetic subjects.

In contradiction to the above series of findings Lawrence *et al.* [1930] and Uberrack & Zell [1931] were unable to recover any insulin from the urine of either normal or diabetic persons, although they employed the same method as Partos. Fisher & Noble [1923] were also unable to detect any insulin in normal urine or the urine of diabetic patients (excepting those who had been treated with insulin), though they demonstrated that the kidney was capable of excreting it by injecting large amounts (40 units) into the circulation of normal dogs under veronal anesthesia and recovering almost the entire quantity from the urine. Bruger & Friedman [1938] have performed similar experiments on rabbits and their results are in agreement with those of Fisher & Noble, as also are those of Goadby & Richardson [1940] who injected 40 units of insulin into rabbits and later withdrew samples of blood at varying time intervals. This blood when injected into another fasting rabbit was found to depress the blood sugar. They considered that this action was due to the injected insulin and found that the hypoglycaemic activity gradually disappeared, being absent after 90 min., although when the kidneys were excluded from the circulation the activity lasted much longer.

These conflicting opinions may well be due to the fact that only a few of the workers have investigated sufficiently critically the methods they have employed to detect the presence of insulin in urine and in only one instance (Goadby & Richardson) do experiments appear to have been carried out to determine the smallest amount of insulin that could be detected by the method adopted. The present paper describes attempts made to find out under what conditions insulin can be recovered from urine and how far such methods can be made quantitative.

Experimental

Detection of hypoglycaemic activity in extracts

A rabbit that had been starved for at least 18 hr. was removed to a box in the laboratory, where it was allowed to rest for 45 min. before the experiment was commenced. The usual precautions were taken to avoid fluctuations of the blood sugar as far as possible.

* Halley Stewart Research Fellow during the early part of this work.

Two 0·1 ml. samples of blood were collected from the marginal ear vein and a known volume of the extract under investigation was injected intravenously. Further 0·1 ml. samples of blood were collected at 10, 20, 30, 45, 60, 75 and 90 min. intervals after the injection and the blood sugars subsequently estimated by the method of Hagedorn & Jensen [1923].

The blood sugar curves given in this paper represent the average response of three or more rabbits. Unfortunately, owing to the war, the number of animals available for this work has been rather limited. If the response to any important preparation was being investigated or if the results were indecisive more rabbits were used. The encircled figure adjoining each curve denotes the number of animals employed.

The smallest quantity of insulin that will give a satisfactory depression curve was found to be 0·2 unit. The response in this case was, on the average, a depression of 25 mg./100 ml. (min. 18, max. 36) in 20 or 30 min. from the time of injection, followed

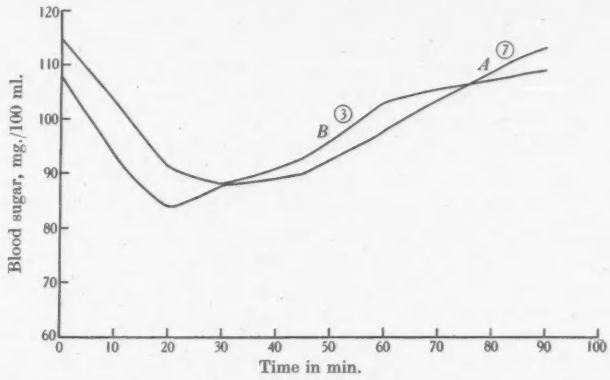

Fig. 1. Curve *A*. Blood sugar curve resulting after injection of 0·2 unit insulin directly. Curve *B*. Blood sugar curve resulting after injection of 5 ml. of 10 ml. extract prepared from normal urine. 0·4 unit of insulin was added to the 10 ml. extract.

by a return to fasting level in 90 min. The average response of 7 rabbits is shown in Fig. 1, curve *A*. Smaller quantities of insulin do not produce satisfactory results, it being unwise to attach much significance to a fall in blood sugar of less than 20 mg./100 ml., as the fasting level may well vary within ± 10 mg. during the 90 min. of the experiment, even when no injection has been made. All the results have been compared with this curve.

Preparation of extracts for injection

Since the presence of insulin in urine is uncertain, in all preliminary experiments small quantities of the crystalline material were added to the urine before commencing the preparation of the extract.

Direct injection of urine. 10 ml. samples of urine (containing 2–10 units insulin per 100 ml.) were injected directly into a number of rabbits. This method was found to be unsatisfactory and was abandoned because the toxic substances present produced an initial rise in blood sugar. The rise was usually followed by a depression to a figure below that of the fasting level. The significance of this fall after the preliminary rise was uncertain and it was deemed that the method would not be a very satisfactory one for detecting small amounts of insulin.

Alcoholic extraction. Insulin is soluble in 80 % aqueous alcohol [Collip, 1923] and this fact was used by Partos [1929] as the basis of his method for extracting it from urine. In

the present investigation extracts were prepared by this method from 100 ml. of urine to which 10 units of insulin had been added. When the entire extracts were injected they produced a marked fall in the blood sugar of the experimental rabbits, but when smaller quantities of the extract were injected it became apparent that only a portion of the insulin had been recovered.

Precipitation of the insulin with ammonium sulphate. Doisy *et al.* [1923] showed that insulin could be fairly completely precipitated from aqueous solution by half-saturation with ammonium sulphate and they made use of this fact in their method for isolating it from fresh pancreas. Fisher & Noble [1923] employed this principle for extracting insulin from urine, but unfortunately their paper does not include any details of technique. This type of method has one considerable advantage in that glucose is not adsorbed on to the precipitate when extracts are prepared from diabetic urines containing sugar. If, however, an alcoholic extraction process such as that of Partos is employed with a urine containing sugar, the sugar is extracted as well and must be removed by fermentation or other means before the fluid can be tested for its power to lower the blood sugar. To assist the precipitation of small amounts of insulin the author added a small quantity of freshly collected rabbit's plasma to the urine so that a bulky precipitate formed which it was hoped would carry down the insulin by entrainment.

Experiments on these lines proved successful and became the basis of the procedure finally adopted which is described in detail below.

Detailed description of method

Reagents and apparatus: Ammonium sulphate (crystalline). A.R. HCl. Boric acid buffer of *p*H 8·0 (prepared by taking 0·62 g. boric acid, 8 ml. *N*/10 NaOH and 65 ml. 0·85 % NaCl and diluting to 200 ml.) [modified from Cole, 1933]. Bromocresol green indicator. Lovibond comparator and disk (for bromocresol green, range 3·6–5·2). Rabbit's plasma freshly collected. Hagedorn and Jensen blood sugar reagents.

The urine was first adjusted to *p*H 5·0 with HCl, using a Lovibond comparator and bromocresol green as indicator. 0·5 ml. of rabbit plasma was added to a 100 ml. sample of urine and this was half-saturated with ammonium sulphate by the addition of 32 g. of the crystalline salt. The mixture was stirred well to bring the salt into solution and the urine was kept in a warm place (about 37°) in order that the resulting precipitate should flocculate.

The precipitate was collected by filtering the urine through a no. 42 Whatman filter paper on a Büchner funnel and allowing to dry (on the filter paper) in a desiccator. This precipitate, which contained the insulin, could be left in the desiccator for at least 4 days without losing its activity. Before assay it was washed off the filter paper with 10 ml. of a borate buffer solution at *p*H 8·0 and then ground up well with this solution in a mortar. Some of the solid material did not dissolve and was removed in the centrifuge. The supernatant fluid containing the active insulin was injected into the test animals.

The effect of p*H on precipitation and elution.* The isoelectric point of insulin is near to *p*H 5·0 [Harrow & Sherwin, 1934]. It was expected therefore, that the optimum *p*H for precipitation would be in the region of that point and this was found to be so in practice. Fig. 2 shows the curves obtained when the precipitation was carried out at varying *p*H. In each case the extract was made from 100 ml. of urine to which 2 units of insulin had been added and 5 ml. of the final 10 ml. extract were injected into the rabbit. It will be seen that the response resulted in a smaller depression of the blood sugar as the *p*H of the urine was lowered.

Elution was effected with a buffer solution at *p*H 8·0. It was considered that this *p*H was sufficiently far removed from that at which precipitation had taken place to dissolve the insulin. Satisfactory results were obtained, but acidification of the solution produced

a small precipitate and it seemed that this might be a possible way of removing further impurities. Accordingly nine extracts were prepared from different normal urines and each dissolved in 10 ml. of buffer solution (*p*H 8·0); 0·4 unit of insulin was added to every

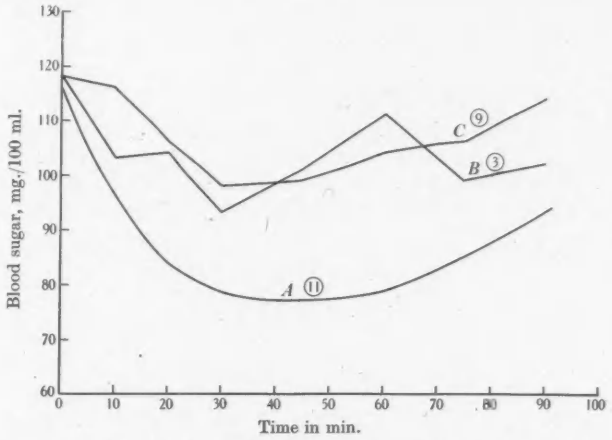

Fig. 2. Blood sugar curves obtained from extracts prepared from urines of differing *p*H. In each case 2·0 units were added to 100 ml. urine before extracting and 5 ml. of the 10 ml. extract injected. Curve *A*. *p*H 5·0. Curve *B*. *p*H 4·0. Curve *C*. *p*H 3·0.

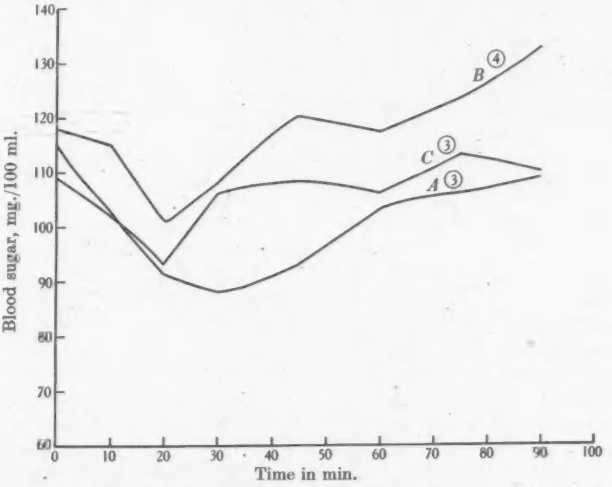

Fig. 3. Blood sugar curves obtained from extracts of differing *p*H. 0·4 unit insulin added to 10 ml. extract prepared from 100 ml. urine and 5 ml. of the extract injected in each case. Curve *A*. *p*H 8·0. Curve *B*. *p*H 4·0. Curve *C*. *p*H 3·0.

one and then three were adjusted to *p*H 4·0, three to *p*H 3·0 and the remaining three were kept as controls. After these adjustments the extracts were kept for 30 min. and were then centrifuged. 5 ml. of the supernatant fluid were used for assay in each case. The

results, which are given in Fig. 3 show that a better response is obtained without acidification. It is probable that a small amount of insulin is carried down with the precipitate especially at *p*H 4·0. The extracts were always left on the filter paper in the desiccator until the day of assay and then they were dissolved immediately before injection. In this way any loss that might occur following the dissolving of the insulin in an alkaline solution was eliminated as far as possible.

Quantitative aspects of insulin recovery

Normal urine without added insulin. If Partos and other workers who think as he does are correct, insulin is a normal component of urine and the extent of recovery of added insulin cannot be ascertained without some knowledge of the total amount present in the original specimen. Extracts were prepared in the above manner from many samples of

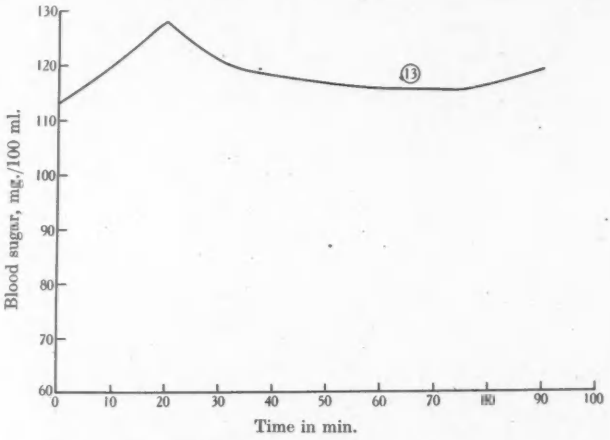

Fig. 4. Blood sugar curve resulting from extracts prepared from normal urine to which no insulin had been added.

urine collected from normal subjects to which no insulin had been added. In some instances 24-hr. specimens were used and at other times the extract was prepared from a fresh 2- or 3-hr. sample. The blood sugar curve representing the average response of 13 rabbits is shown in Fig. 4. The curves almost invariably showed a small rise during the first 20 min. and in only one instance was there any depression of the blood sugar. From these results it seems unlikely that there is any insulin in normal urine.

Normal urine with added insulin. Small quantities of protein and other substances in the extract besides insulin might alter the response of the rabbit to 0·2 unit insulin, but in practice this was not found to be the case. 0·2 unit was added to half (5 ml.) the extract prepared from 100 ml. normal urine and this was injected into a rabbit. The average response obtained from three such experiments differs little from the effect of injecting 0·2 unit directly (Fig. 1, curves *A*, *B*) and either curve can be used for comparison with those resulting from attempts to recover insulin added to urine.

Experiments were now carried out in order to assess the amount of insulin that was recovered by this method when 2 units were added to 100 ml. of urine. Extracts were prepared in the manner described above and the volume of fluid for injection made up to 10 ml. Then 5, 2 and 1 ml. (equivalent to theoretical recoveries of 1, 0·4 and 0·2 unit respectively) were injected into different rabbits. Fig. 5 shows the responses obtained and

if the curve *C* is compared with those in Fig. 1 it is apparent that most of the insulin has been recovered. However, it was hoped that it would be possible to recover quantities smaller than 2 units/100 ml. and so specimens of urine containing 0·5 and 0·25 unit/100 ml. were taken and extracts prepared from them. Half the extract from the former (i.e. equivalent to 0·25 unit) and the entire extract from the latter (made up to 5 ml. only in

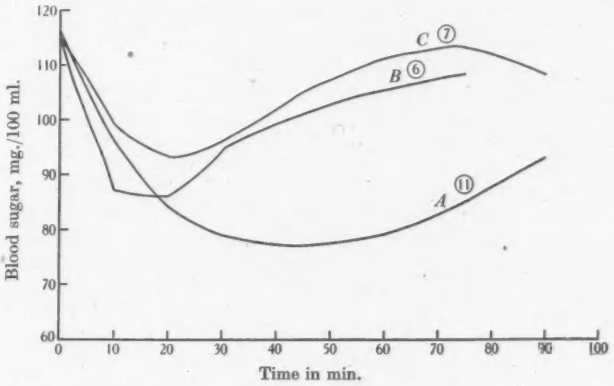

Fig. 5. Blood sugar curves resulting after injecting varying quantities of a 10 ml. extract prepared from 100 ml. urine to which 2·0 units insulin had been added. Curve *A*. 5·0 ml. Curve *B*. 2·0 ml. Curve *C*. 1·0 ml.

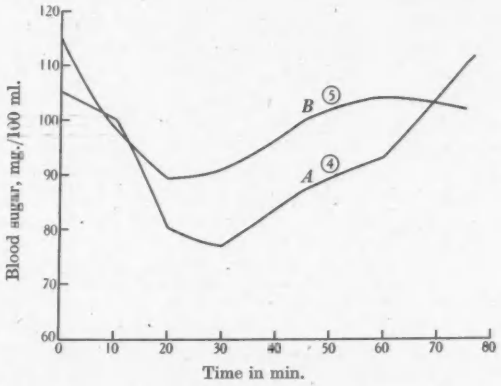

Fig. 6. Curve *A*. Blood sugar curve obtained after injecting half extract prepared from 100 ml. normal urine to which 0·5 unit insulin had been added. Curve *B*. Blood sugar curve obtained after injecting entire extract prepared from 100 ml. urine to which 0·25 unit insulin had been added.

this case) were used for assay. Thus, in theory, each should contain the same amount of insulin. The results are shown in Fig. 6 and it will be seen that the two curves are very similar, the response in the first case being a maximum depression of 28 mg. and in the second case 26 mg., reached in 30 and 20 min. respectively. Again, these two curves are very similar to those shown in Fig. 1 and suggest that most if not all, of the insulin is recovered by this technique and, furthermore, that it is possible to detect the presence of insulin in urine, even when it is there in quantities as small as 0·25 unit/100 ml.

Stability of insulin in urine

In all the experiments so far described, the extracts have been prepared immediately after the addition of insulin to the urine. When collecting specimens from human subjects it is not always possible to commence the extraction immediately after the specimen has been voided and this is, of course, particularly the case when 24 hr. specimens have to be examined for the presence of insulin. The question arises as to whether the insulin becomes inactivated on long standing in urine. If this were so it would be a possible explanation of the varying results obtained by the earlier workers.

This possibility has been examined in the following ways. Several 24 hr. specimens were collected by different normal subjects but each specimen that was passed was halved immediately. To one half insulin was added straight away (the amount varied from 0·5 to 2 unit/100 ml. in the different collections). No insulin was added to the other half, which was used as a control. Extracts were prepared from both portions as soon as the 24 hr. collection had been completed. When half the extract from the control was injected the blood sugar did not fall, whereas when the other extract was investigated by employing an amount for injection that would theoretically contain 0·25 unit, the rabbit's blood sugar showed on the average a fall of 25 mg. within 30 min. (see Fig. 7, curve *A*, which shows a typical response) indicating that no apparent loss of insulin occurs during the collection of a 24 hr. sample when this is analysed immediately. Some of these specimens were left in the ice chest for 5–7 days and then a second extract prepared, of which the same quantity was used for injection. Fig. 7 shows the result of one such experiment. In this case the original specimens contained 0·5 unit/100 ml., and the extracts were made up to 10 ml., of which 5 ml. were used for injection. Curve *A* shows the effect when the extract was prepared immediately, whereas curve *B* shows the response that is obtained if the specimen is kept in the ice chest for 5 days before commencing the extraction and it is apparent that some loss has taken place.

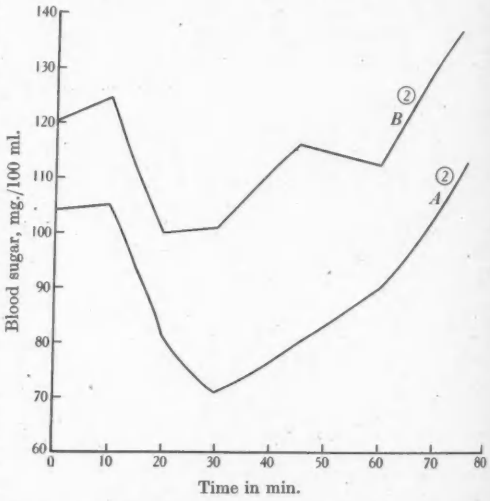

Fig. 7. Curve *A*. Blood sugar curve resulting from injection of 5 ml. of 10 ml. extract prepared from 24 hourly specimen of urine containing 0·5 unit insulin per 100 ml. Curve *B*. Response after injecting 5 ml. of 10 ml. extract prepared from the same urine 5 days later.

In other experiments in which varying quantities of insulin (0·2–2 units/100 ml.) have been added to specimens of normal urine, which have been kept for 24 hr. at room temperature before commencing the extraction of insulin, there does not appear to be any measurable loss of activity. This is probably the maximum length of time that it is safe to leave the specimen before commencing the ammonium sulphate precipitation and subsequent extraction of insulin.

Finally there is a possibility that insulin may be destroyed in the urine while in the bladder, where the higher temperature might lessen its stability. Accordingly specimens

of urine containing 2 units/100 ml. were incubated at 37° for 1 hr. before commencing the extraction. Assays were made and showed that there is little likelihood of any loss due to this cause *in vivo*, as curves identical with those shown in Fig. 5 were obtained.

Conclusions

The experiments described above have shown that it is quite possible to detect the presence of 0·25 unit of insulin in 100 ml. urine even 24 hr. after it has been voided. On the other hand when extracts were prepared from normal urine to which no insulin had been added and injected into fasting rabbits, no fall in blood sugar resulted. This suggests that insulin is not a component of normal urine, or if it is, must be present in quantities smaller than 0·25 unit/100 ml.

Up to the present it has not been possible to investigate the urine of either untreated diabetic persons, or diabetics receiving insulin, excepting one patient who was very resistant to insulin therapy and was having 240 units of insulin per day. Two 24 hr. specimens were collected from this patient and examined in the manner described above but no insulin was detected in either. It is not possible to draw any conclusions from this one case but it is hoped it will be possible to carry out more work along these lines and make this the subject of a future communication.

Summary

1. A method for detecting the presence of insulin in urine has been described and investigated.

2. It has been shown that it is possible to detect the presence of 0·25 unit insulin per 100 ml. when this is added *in vitro*.

3. Insulin added to urine could be recovered 24 hr. later without apparent loss of activity, nor was the activity destroyed by incubating at 37° for 1 hr.

4. No insulin could be detected in normal urine and, if present, must be there in amounts less than 0·25 unit/100 ml.

I should like to express my thanks to Dr C. H. Gray for suggesting and supervising this work and to Dr J. Patterson for his many helpful suggestions and encouragement. I should also like to thank the Halley Stewart Trust for a personal grant. The rabbits used in these experiments were paid for out of a grant from the Medical Research Council to the diabetic clinic of King's College Hospital for research on diabetes.

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43. Haemopoiesis in Lead Poisoning

By **J. E. Kench,*** **A. E. Gillam** and **R. E. Lane**, *From the University of Manchester*

(*Received 16 March 1942*)

The marked changes in blood resulting from lead poisoning were ascribed by Aub *et al.* [1924] to increased destruction of the red blood corpuscles. From *in vitro* experiments these workers concluded that the cells became more fragile when exposed to traces of lead and consequently were less able to withstand the trauma to which they were exposed during circulation. This view receives support from Key [1924] and Brookfield [1928], and from the interesting observations of Clowes [1916] and of Bhatnagar [1921] on the effect of electrolytes on lipoid-water emulsions. Small ionic concentrations of heavy metals produced remarkable changes in the stability of such emulsions, which Clowes has discussed in detail from the viewpoint of the cell wall and its permeability.

In the last few years an additional approach has been possible through our increased knowledge of the porphyrin pigments and their relation to haemoglobin. Differences between lead anaemia and acute haemolytic anaemia have thus been brought to light, and Rimington [1938] has suggested that lead inhibits haemoglobin synthesis by preventing the incorporation of iron into the protoporphyrin nucleus. The pigment so set free appears in the urine as coproporphyrin III. This latter pigment may be found in the urine in lead poisoning when there is no evidence of increased blood destruction (i.e. the faecal urobilinogen is not increased).

Increase of blood protoporphyrin in lead poisoning has been reported by Vigliani & Waldenström [1937], and of non-haemoglobin iron by Vannotti & Imholz [1939], which induced these authors to conclude that the blocking theory had been substantiated. It is readily apparent, however, that the increase of non-haemoglobin iron observed by these workers (approximately 1 mg./100 ml.) is inadequate to account for the 25 % reduction in haemoglobin which occurred, for which the iron supply to the bone marrow would be 13 mg./100 ml. Similarly, the quantities of free protoporphyrin and coproporphyrin III reported as produced by lead intoxication (of the order 0·0003 g.) are extremely small compared with the normal daily production of protoporphyrin (0·5 g.) necessary to replace the haemoglobin changed into and excreted as bile pigments.

The investigations described in this paper were undertaken to obtain more information on the changes in haemopoiesis caused by lead, especial attention being paid to the blood and urinary porphyrins, and their relation to cell production.

Experimental

Specimens of urine were collected from lead workers over a period of 3 weeks. 100 ml. aliquots were taken for determination of lead by a modified dithizone method [Kench, 1940], and the ether-soluble porphyrins in the remainder of each specimen were separated during 12 hr. continuous extraction by the ether-acetic acid method [Rimington & Hemmings, 1939].

Venous blood samples were also taken and protoporphyrin extracted from separated cells and plasma by the method of Van den Bergh & Hyman [1928]. The relative volumes of cells and plasma were found by the haematocrit, whilst haemoglobin values (taking as the normal or 100 %, 14·5 g. haemoglobin/100 ml.) were obtained photometrically after conversion into globin haemochromogen (checked against iron and Haldane gasometric methods). Blood lead values were determined on 5–10 ml. of each specimen by drying

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and charring on the hot plate, and digesting with the minimum volume of lead-free H_2SO_4 (usually 10–20 ml.) required to produce a colourless digest. Lead was separated as its complex with sodium diethyl dithiocarbamate in ether, and finally determined as dithizonate in chloroform, using a Hilger Spekker micro-absorptiometer and a green filter (transparent 520–540 mμ).

Blood films were stained by alkaline methylene blue, and red cells showing punctate basophilia were enumerated by Seller's technique, using transmitted light, as described by Lane [1931].

For the final determination of porphyrins a Hilger-Nutting spectrophotometer was employed, the absorption measurements on the acidified extracts being carried out at 549 mμ for coproporphyrin and 558 mμ for protoporphyrin, at which wave-lengths these pigments exhibit intense and highly selective absorption bands.

The relative values were calculated to absolute concentrations of porphyrin from absorption data first obtained on standard solutions of the pigments. Coproporphyrin I standard in 0·5 % HCl was kindly supplied by Dr C. Rimington; it contained 10 μg./ml. and showed an intensity of absorption in a 4 cm. cell $E = \log I_0/I = 1{\cdot}047$, giving a standard value of $E^{1\%}_{1\,\text{cm.}}$ (549 mμ) = 262.

Protoporphyrin was prepared from pyridine haemochromogen by reduction with hydrazine hydrate and treatment with conc. HCl, according to the method of Fischer & Pützer [1926]. The crude pigment was converted into the dimethyl ester by dissolving in methyl alcohol and saturating the solution at 0° with dry hydrogen chloride. After standing for 24 hr. at 0–4°, the methyl ester was extracted with chloroform and purified by fractional crystallization from dry benzene, the purity of each fraction being tested with the Hilger-Nutting visual spectrophotometer. The purest crystals dissolved in 20 % HCl had $E^{1\%}_{1\,\text{cm.}}$ (558 mμ) = 240, and this value was used as standard.

After the quantitative analyses were completed the urinary coproporphyrin concentrates were pooled for the preparation of the corresponding methyl ester. This proved to have the crystalline appearance, solubility and double melting point (149°, remelt 172°) characteristic of the series III isomer.

Results and discussion

The first investigation was made on groups of lead workers thought likely to provide information on the relationship between haemoglobin concentration and urinary coproporphyrin excretion. Although it was found impracticable to examine faecal coproporphyrin, the findings of Watson [1937] show that coproporphyrin III excretion is confined almost entirely to the urine. We have assumed therefore that the urinary coproporphyrin values represent practically the total loss of series III porphyrin.

The data are presented in Table 1. The first group includes men who at the time of examination were exposed to moderately heavy lead absorption, e.g. the first subject had developed anaemia to the extent of 13 % in 6 months. The punctate count was the lowest and coproporphyrin excretion the highest of the group. On the other hand, the second subject, with a high punctate count and low urinary coproporphyrin level, showed no change in circulating haemoglobin. Subject no. 12 had long-established anaemia with a very low rate of regeneration, and it has been found impossible to raise the haemoglobin level by iron therapy.

If anaemia were due entirely to prevention of iron-porphyrin formation in accordance with Rimington's hypothesis we should expect approximately one-quarter of the protoporphyrin produced, viz. 0·12 g. to be excreted. Actually, we find only 0·00024 g., and in all cases coproporphyrin excretion represents but an extremely small and variable fraction of the total pigment formed, and its magnitude is independent both of the number of stippled red cells and the degree of anaemia. Moreover, this is true also of the free protoporphyrin found in the blood during active absorption of lead.

Table 1. *Haemoglobin values, punctate counts and urinary coproporphyrin excretion in lead workers*

Lead worker	Duration of exposure yr.	Av. Hb % (Haldane)	Hb changes %	Av. punctate count per 10^6 R.B.C.	Urinary lead μg./l.	Av. urinary coproporphyrin III μg./1500 ml.
1	½	84	−13	4000	295	515
2		91	0	7000	182	25
3		93	− 6	6500	324	164
4		91	−10	5500	354	86
5	10	100	None	1000	303	51
6		95		2000	234	181
7		86		3000	262	62
8		85		1500	197	24
9		94		5000	296	111
10		96		3000	340	60
11		91		1000	287	82
12	20	76	None	Free	436	238

Table 2. *Blood protoporphyrin during oral administration of lead acetate*

Duration of exp. days	Lead μg./100 ml.		Protoporphyrin μg./100 ml.		Punctate basophilia per 10^6 R.B.C.	Urinary coproporphyrin μg./day
	Cells	Plasma	Cells	Plasma		
1	50	47	56	52	1000	22
8	84	56	40	139	2500	134
17	32	159	56	54	2000	268
23	50	128	56	140	4000	—
28	207	157	56	130	2000	232
33	34	178	50	170	3000	—
38	86	75	65	68	1500	—

Table 3. *Lead and protoporphyrin in the blood of lead workers*

Case	Duration of lead exposure yr.	Blood lead μg./100 ml.		Protoporphyrin μg./100 ml.		Urinary copropor-phyrin III μg./l.	Hb % (Haldane)	Punctate count per 10^6 R.B.C.
13	½	204		271		646	70	5,000 S, M, L
14		70		75		413	80	5,000 S, M
15		67		122		426	89	Free
16	4	74		428		81	100	1,500 S, M
17	16	70		48		356	85	2,500 S, M
18	17	58		95		60	97	1,000 S
19	21	76		103		308	101	Free
20	28	188		216		526	92	1,500 S, M
21	26	130		232		245	86	4,000 S, M, L
				Cells	Plasma			
22	2	268		33	44	—	105	2,000
23	2	305		24	34	—	81	15,500 S, M, L
		Cells	Plasma					
24	2	31	22	34	30	—	77	15,000 S, M, L
25	17	57	23	65	38	—	97	12,000 S, M, L
26	10	98	29	90	14	—	86	Free

S, M, and L denote small, medium and large granules respectively.

Blood porphyrin changes in J. E. K. were observed during oral administration of 20 mg. of lead acetate daily (Table 2) and can be summarized briefly as follows:

(1) The concentration of protoporphyrin was generally greater in the plasma than in the cells.

(2) The basophilic substance of reticulocytes has been identified by Watson & Clarke [1937] as protoporphyrin; but intracellular protoporphyrin in this and other experiments was independent of the number of reticulocytes and of erythrocytes showing stippling.

(3) No change in haemoglobin concentration in the blood could be detected throughout, but urinary coproporphyrin III excretion reached a daily average value of 268 μg.

Finally, we have examined the possible relationship between the concentration of lead in the blood and the protoporphyrin content. The results, which appear in Table 3, indicate that (1) the number of stippled red cells gives no indication of the protoporphyrin content of the whole blood; (2) the concentration of the porphyrin in blood is greater than that of the coproporphyrin in the urine, but there is no constant numerical relationship between the pigment levels; (3) the ratio free/combined protoporphyrin in the blood is extremely small in all cases.

Consideration of these results leads to the following conclusions: (I) The physiological action of small amounts of lead cannot be explained in terms of partial prevention of the iron-porphyrin complex formation. (II) The occurrence of protoporphyrin and non-haemoglobin iron to the extent observed in these and other experiments appears to be only incidental to generally restricted cellular activity. Erythrocytes are hypochromic and reduced in numbers according to the degree of inhibition, in contrast to the findings in uncomplicated haemolysis in which no cellular poisons are present.

In the light of these results it seems to us that the following explanation is a more acceptable one. The immediate action of lead after its passage into the blood stream appears to be adsorption on the lipoid-protein film which constitutes the red cell membrane. Ørskov [1935] observed rapid shrinkage of cells exposed to lead and rapid loss of potassium into the surrounding medium. At pH 6·3–6·6 and bicarbonate ion 0·007 N a concentration of lead of 1 in 25,000,000 caused a marked increase in the permeability of red cells to potassium. These changes could only be observed in those mammalian species in which anaemia and stippling are predominant signs of lead poisoning and are of fundamental importance in the injurious action produced by lead.

In the early stages of lead exposure the destruction of cells in the peripheral blood leads to augmented formation of breakdown products which serve to stimulate the bone marrow. This activity, however, may, after continued lead absorption, be considerably depressed by the presence of a comparatively high concentration of lead in the bone marrow, and sternal puncture as carried out by one of us (R. E. L.) has confirmed this. Vannotti & Siegrist [1940] have shown recently that in bone marrow cultures taken from lead-intoxicated animals, haemoglobin production was inhibited, but returned to normal after addition of lactoflavin. The findings of Holmes *et al.* [1939] suggest that diminution of haemopoiesis in many cases may be explained by a deficiency of available ascorbic acid. During chronic lead poisoning an increased intake of ascorbic acid was required in order to overcome the inhibiting effect of traces of lead on intracellular reactions. This may be due to complex ion formation, investigated recently by Kety [1942] by potentiometric methods. Citrate, lactate, acetate and ascorbate ions all combine with lead to form soluble complexes whose dissociation was measured. The citrate complex is the least dissociated, and administration of citrate has been recommended, therefore, to increase mobilization and excretion of lead without increasing the concentration of toxic lead ions.

Summary

A study has been made of blood and urinary porphyrins in lead workers in order to ascertain how lead affects haemopoiesis. Our conclusions are:

I. The physiological action of small amounts of lead cannot be explained in terms of partial prevention of iron-porphyrin complex formation.

II. The occurrence of protoporphyrin and non-haemoglobin iron to the extent observed in these and other experiments appears to be incidental to restricted cellular activity.

III. Diminution of haemoglobin in lead poisoning is a consequence, not of non-utilization of protoporphyrin, but of restricted formation of this vital pigment.

One of us (J. E. K.) is indebted to the Sir Halley Stewart Trustees for a grant and to the Director of the Department of Clinical Investigations and Research, Dr J. F. Wilkinson, for his interest and support.

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44. Metabolism of Steroids

3. The 7-Hydroxycholesterols as Metabolites

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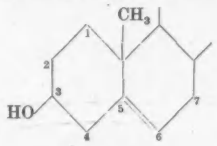
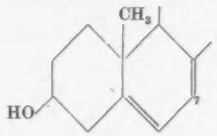
The work of Wintersteiner & Ritzmann [1940] and of Bergström & Wintersteiner [1941] has demonstrated the presence of both α- and β-7-hydroxycholesterol in various tissue extracts, and has indicated that these cholesterol oxidation products might arise, in part at least, during the isolation processes. It is clear also that there exist in the body the conditions (presence of cholesterol in fine suspension and of O_2, and an alkaline reaction) which the American workers have shown to result in formation of 7-hydroxycholesterol *in vitro* [Bergström & Wintersteiner, 1941]. The conclusion is that these substances may in fact be present in the blood and other tissues, and this conclusion has now been further tested, first by attempts at the isolation of 7-hydroxysterols from plant sources (yeast fat, acorn fat and wheat germ oil), and secondly by the saponification of cholesteryl stearate.

In the plant fats, no substances of the type under discussion were detected. Although, especially in a complex mixture such as yeast fat, 7-hydroxysterols may have escaped notice, it nevertheless seems reasonable to deduce that such sterols, if present at all, exist only in very small amounts, much smaller than are found in ox liver. The sitosterols of wheat germ and acorn fat are believed to contain the cholesterol nuclear structure (I) and are readily oxidized to what appear to be 7-hydroxy compounds by methods such as those used by Bergström & Wintersteiner [1941] for cholesterol:



(I)



(II)

It is not known whether sterols of type (I) exist in yeast. Pure 'synthetic' cholesteryl stearate was saponified under the conditions used for the isolation of 7-hydroxycholesterol from ox liver. No oxidation products of this type could be detected. It therefore seems reasonable to conclude: (1) that if 7-hydroxycholesterol is formed during isolation procedures it is formed in very small amounts; (2) that most of the 7-hydroxycholesterol found in tissues is to be regarded as a cholesterol oxidation product existing there *in vivo*. There is as yet no experimental evidence whatever to show that the presence of 7-hydroxycholesterol is connected with the formation of 7-dehydrocholesterol (II), the precursor of vitamin D_3. Examination of a tunny liver oil of high vitamin D assay showed the presence of only minute amounts of sterols of the 7-hydroxy type.

The methods used in this work were those previously described [Haslewood, 1939; 1941]. The isolation of digitonin-precipitable material, insoluble in light petroleum, and giving an intense blue colour with antimony trichloride [Barr *et al.* 1936], was taken as evidence of the presence of 7-hydroxysterols.

Experimental

General methods. The unsaponifiable portion of the fats was dissolved in alcohol and the precipitate of 'sterols' filtered off. The liquors, on evaporation, left Fraction A. This was fractionated between 90% methyl alcohol and light petroleum (l.p.), B.P. 40–60°,

as previously described [Haslewood, 1941], the l.p.-soluble containing Fraction B and the alcohol-soluble, Fraction C. Fraction C is cholesterol-free and readily yields 7-hydroxycholesterol in the case of ox-liver fat. Fraction B was further fractionated as described; when the starting material was ox-liver, this fraction also contained hydroxycholesterol.

Yeast fat. Starting material (supplied by Glaxo Laboratories) was the residue from the separation of ergosterol. When this (100 g.) was allowed to stand with benzene (300 ml.), there separated a gelatinous solid which gave negative colour reactions. 30 ml. of the benzene filtrate were run through an Al_2O_3 column (Merck: diam. 1·2 cm., length 16 cm.), and the column was washed with 400 ml. of benzene. Little or no digitonin-precipitable material was obtained after elution of the top half of the column. In other cases, the whole column was eluted with alcohol and acetone and the eluate, with digitonin in 80 % alcohol and a little ether, gave 30–70 mg. of digitonide showing a blue colour of variable intensity with $SbCl_3$ in chloroform. Decomposition of 0·27 g. of the accumulated digitonide with pyridine and ether yielded oily material, of which approximately 30 mg. were insoluble in l.p. and gave a faint colour with $SbCl_3$. No solid was obtained after benzoylation. Many experiments such as the above resulted only in the preparation of very intractable and apparently unstable gums, which may have contained some 7-hydroxysterols: this is, however, unlikely, for it is to be expected that these substances will be adsorbed from benzene solution on the top part of the column [Haslewood, 1939]. When the adsorption was carried out as above and the benzene was collected separately after the first drops leaving the column had become colourless, there was obtained on evaporation of this benzene a crystalline solid which was also part of the material which separated (see below) during the 90 % methyl alcohol-l.p. fractionation. This substance, after recrystallization from dilute alcohol and then benzene, formed almost colourless needles, M.P. 110–111°. (Found (by Dr H. Nisbet, Edinburgh): C, 69·7; H, 11·3 %. Mol. wt. (Rast) = 390. $(C_5H_{10}O)_n$ requires C, 69·8; H, 11·6 %.) This compound appeared to be an alcohol.

Variable amounts of solid material separated when the original fat was shaken with 90 % methyl alcohol and l.p. 2 g. of this solid were shaken with benzene and the benzene-soluble portion, in benzene, was run through an Al_2O_3 column (1·2 × 10 cm.) and washed with 250 ml. of the solvent. Evaporation of the benzene gave 0·14 g. of impure digitonin-precipitable 'sterol', M.P. 134–145°, giving a benzoate M.P. 161–165°, not affected by boiling dimethylaniline (30 hr.). The substance and benzoate gave positive Liebermann-Burchard and Tortelli-Jaffé reactions, and intense bluish-purple colours with $SbCl_3$. Fraction C (1–2 g. from 20 g. of fat, after removal of about 1 g. of the above solid and of benzene-insoluble material) yielded 0·2–0·3 g. of digitonide which gave no colour with $SbCl_3$ and no identifiable product after decomposition.

Acorn fat. Peeled, minced and dried acorns (from the English oak, *Quercus Robur* L.) were powdered and extracted in a Soxhlet apparatus with ether until the extract was colourless. Evaporation gave the fat as a dark oil. Yield, 150 g. from 4·75 kg. of dried acorn powder.

This material in alcohol (400 ml.), with KOH (50 g.) in water (50 ml.), was boiled under reflux for 6 hr. The mixture was cooled, diluted to 1900 ml., almost saturated with NaCl and extracted 4 times with ether. Evaporation of the washed ether gave the unsaponifiable matter as a yellowish solid (2·2 g.). The 'sterols' crystallized from methyl alcohol as white plates (1 g.) of M.P. 117–130°, which, by the method of Windaus [1906; cf. Zaki & Soliman, 1940], gave no stigmasterol tetrabromide. The recovered, debrominated and recrystallized acetate had M.P. 119–121°, and the sitosterol obtained from this by saponification melted at 132–134°. The 'sterol' fraction also contained a small quantity of what appeared to be a hydrocarbon, M.P. 65°.

Fraction A (1·2 g.), dissolved in l.p., was run through an Al_2O_3 (Hopkin and Williams) column (0·8 × 15 cm.) and the column was washed with 250 ml. of l.p. 20 mg. of digitonide

were isolated after elution of the top third of the column. In re-adsorptions, the whole of the adsorbed material was eluted. No fraction giving a perceptible colour with $SbCl_3$ was isolated from any of the digitonides or eluates obtained. Acorn fat in the amounts examined contained no detectable 7-hydroxysterols.

The above sitosterol (50 mg.) in acetone was added to 5 ml. of Castile soap solution (1 g./100 ml.) and the emulsion heated, with shaking, on the water bath until the smell of acetone had disappeared. After 12 hr. at approximately 80°, with occasional shaking, the mixture was acidified and extracted with ether. Evaporation of the ether, after washing with NaOH and water, left a gum which with l.p. yielded 5 mg. of crystals (probably impure 7-hydroxysitosterol) of M.P. 145–149°, giving an intense blue colour with $SbCl_3$. A similar fraction was obtained in the same way from stigmasterol.

Wheat germ oil. Fraction A was supplied by Glaxo Laboratories. Fraction C from 10 g. (corresponding to about 20 g. of 'sterols') of Fraction A weighed 0·25 g. and yielded 40 mg. of a digitonide mixed with a yellow pigment that gave an intense $SbCl_3$ reaction. Decomposition of the digitonide gave a residue completely soluble in l.p. and yielding on re-precipitation with digitonin a small amount of a product not chromogenic with $SbCl_3$.

Fraction B, or the original oil (5 g.), in l.p., was adsorbed on an Al_2O_3 (Hopkin and Williams) column (0·8 × 20 cm.), which was then washed with 500 ml. of l.p. The digitonide obtained after elution of the top third of the column strongly adsorbed the above pigment and on decomposition gave material entirely soluble in l.p. In chloroform, this mixture gave an intense purplish colour with $SbCl_3$; it appeared to be similar to the corresponding fraction from yeast fat. Washing of the whole column with ether, a procedure which had given 7-hydroxycholesterol from ox liver fat [Haslewood, 1941], yielded a fraction from which were prepared 1·2 g. of digitonide, by addition of successive amounts (0·5, 0·3, 0·2, 0·2, and 0·2 g.) of digitonin, the precipitates being collected separately and tested with $SbCl_3$. The first 0·8 g. of digitonin gave 1·0 g. of digitonide not chromogenic with $SbCl_3$, and leaving, after decomposition, a sterol of M.P. 134–137°. The remaining 0·2 g. of digitonide was chromogenic with $SbCl_3$; the sterol from it was soluble in l.p. and similar to the fraction from the top part of the Al_2O_3 column, the $SbCl_3$ test showing a green to blue-green colour.

Saponification of cholesteryl stearate. Cholesteryl stearate (10 g., M.P. 84–85°, prepared from cholesterol and a pyridine solution of redistilled acid chloride, from stearic acid of M.P. 70–71°) was boiled for 5 hr. under reflux with KOH (10 g.) and 50 % alcohol (150 ml.), and allowed to stand for 20 hr. Examination of the unsaponifiable material (prepared as usual after ether extraction) by the methods mentioned gave only cholesterol, no 7-hydroxycholesterol being detected.

Summary

In a careful examination of yeast fat, acorn fat, wheat germ oil and the neutral fraction from saponified cholesteryl stearate, no 7-hydroxysterols could with certainty be detected. It is concluded that these sterols are formed, if at all, only in very small amounts during isolation procedures. The 7-hydroxycholesterols are therefore probably to be regarded as 'genuine' intermediates in cholesterol metabolism.

The author expresses his sincere thanks to Dr E. L. Smith, of Glaxo Laboratories, for generous gifts of yeast fat residues and of wheat germ fractions.

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45. Digestion of Straw by the Ruminant

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(*Received 27 March 1942*)

In the course of an investigation into the possibility of improving the digestibility of straws by biological pretreatment with cultures from the sheep's rumen, it became evident that no very clear picture was available, from previous studies, of the process of ruminant digestion of untreated straws.

The literature contains numerous references to digestibility trials on a variety of straws. The results are, for the most part, presented in terms of such fractions as crude fibre, nitrogen-free extractives etc. Criticisms of this system of analysis are very numerous in the literature of recent years. The fractions isolated by no means represent individual chemical substances, and since, as is the case with straw, one fraction (nitrogen-free extractives) may include a highly digestible substance (hemicellulose) and a relatively indigestible one (lignin), the figures obtained can only be the resultant of two separate processes and are therefore valueless if an accurate study of the digestion process is required. Further, digestibility trials present the net result of all digestive processes which take place between the mouth and the anus and give no indication of whether failure to digest fodder constituents is due to innate causes, to their chemical or physical condition in the plant, or to unsuitable conditions of digestion in the animal (e.g. too rapid passage through the gut). It was therefore thought desirable to study the digestion of the principal nutrients of straw both in the isolated condition and as present in the straw, with special reference to the rates of digestion in each case and the possible degree of digestion if the process were allowed to continue for long periods.

Rumen digestion was studied in most of the experiments. Caecal digestion is usually regarded as of lesser importance in the ruminant. Trautmann & Asher [1939] showed that cellulose introduced into the abomasum or caecum underwent little or no digestion; straw incubated *in vitro* with sheep's caecal contents likewise underwent relatively little digestion (16 % in 7 days).

Methods

In vitro digestions were carried out either with the fluid part of sheep's rumen ingesta, withdrawn through a rumen fistula and filtered through muslin, or with a heavy inoculum of this material diluted with an inorganic bacterial medium consisting of phosphates of sodium and potassium with ammonium, magnesium and calcium salts. In either case $CaCO_3$ was added as a buffer. At first N_2 was bubbled through, but it was found that there was no apparent difference in the rate of digestion when this was omitted; it was therefore assumed that the CO_2 produced during fermentation in the deep fluid kept the concentration of O_2 sufficiently low.

In vivo digestion was observed by suspending weighed portions of the material, in small silk squares tied up with silk on the end of a long silk cord, in a sheep's rumen through a fistula. After varying periods of digestion bags were removed and the contents washed, dried and weighed. The material was previously passed through a domestic mincer, which should well simulate the chewing action of the animal, and was then well washed on muslin to remove any fine particles which might otherwise escape through the pores of the silk. The squares were tied up loosely so that the fluid ingesta might make rapid contact with their contents; however, in spite of these various precautions, it is probable

that the conditions of digestion in the bag were not as favourable as in the rumen, so that the observed rates of digestion must be regarded as minimal.

Cellulose was determined by the method of Norman & Jenkins [1933] which comprises alternate treatments with neutral hypochlorite and sulphite, followed by alternate acid hypochlorite and sulphite. Norman stresses the importance of including in the cellulose determination the xylan which is very closely associated with it. Astbury *et al.* [1935] quote X-ray evidence in support of this close association. The true cellulose is obtained by subtracting the xylan, found by furfuraldehyde determination, from the 'native cellulose' found by Norman's method. An alternative method was originated by Williams & Olmstead [1935]. After removal of starch and digestible protein with an enzyme mixture, they determined the cellulose in the form of fermentable sugar in an acid hydrolysate of the residue, and carbohydrate other than cellulose, without differentiation, as non-fermentable sugar in the hydrolysate. This method, in which no isolation of chemical individuals is attempted, would seem to be of value in large-scale routine sampling, but is not applicable to the present investigation.

The isolation of the constituents of straw containing pentose and uronic acid is a matter of some difficulty. Alkali extraction is employed for obtaining material for studies of constitution, but it is unsatisfactory as the basis of an analytical method. Weihe & Phillips [1940] have studied the constitution of the hemicelluloses of wheat straw and conclude that Fraction B predominates and that it is composed of uronic acid: *l*-arabinose:*d*-xylose in the proportions of 1:0·9:23 units per molecule. As, furthermore, the pentosan associated with cellulose is, according to Norman, a xylan, it was thought that no serious error would arise if pentosan-polyuronide carbohydrates were calculated using the factor given by Tollens-Elsner [1935] for obtaining xylan from furfuraldehyde determined by analysis. Furfuraldehyde obtained by distilling the material with 12% HCl was estimated by bromination with acid sodium bromide-sodium bromate mixture and titration of iodine liberated from potassium iodide by excess bromine, with sodium thiosulphate [Powell & Whittaker, 1924].

A discussion of the chemistry of lignin, a subject of considerable controversy, would be out of place here except in so far as it affects the methods by which lignin may be estimated in straw. The standard property by means of which lignin is isolated is its insolubility when the material is kept in cold 72% H_2SO_4 for 24 hr. and then diluted. Norman & Jenkins [1934] pointed out that certain sugars, including xylose and arabinose, but excluding glucose, give rise, through the production of furfuraldehyde, to insoluble products when in contact with lignin and strong H_2SO_4 or HCl. They therefore recommend the removal of pentosans by dilute acid hydrolysis before treating the residue with strong H_2SO_4. Hilpert & Littman [1935] question whether the product of strong acid treatment represents a substance which had a separate chemical identity in the plant material. This criticism was largely based on their claim that, at $-10°$, 72% H_2SO_4 dissolved the whole of the organic material of straw. The familiar brownish 'lignin' only appeared when the solution was allowed to become warm; according to them it was merely an artefact of carbohydrate origin. They make no attempt to suggest what, if lignin as such does not exist, is the nature of the substance which gives the typical microscopic reactions of lignin in the intact plant, or what substance fills the gap in the percentage composition of straw when the sum is made of all known constituents.

The standard method with Norman's modification has thus been adopted, since it is thought that if the product weighed has not the same composition as the lignin in the straw, it does at least represent the lignin quantitatively and digestion of the lignin will result in diminution of the yield of the substance isolated by strong acid treatment. Other straw constituents are of minor importance from a quantitative point of view.

Digestion of whole straw

The rate and degree of digestion of whole oat and wheat straws were first studied by the *in vivo* method already described. *In vitro* studies were also made, but owing to the long period of digestion the conditions could not be so well controlled and the results tended to be more variable. Maximum digestion achieved *in vitro* was 50%.

Table 1. *Digestion of wheat straw* in vivo (*0·5 g. samples*)

	Period of digestion, days					
	1	2	3	4	5	7
% loss in weight: (A)	22·9	35·9	40·0	47·5	48·2	60·5
(B)	8·4	27·0	32·1	25·8	43·5	41·9
(C)	14·4	—	32·5	—	46·9	33·1

Average maximum digestion in experiments (6) lasting 6 days or more was 48·6%.

Digestion of oat straw in vivo (*0·5 g. samples*)

	Period of digestion, days						
	1	2	3	4	5	6	7
% loss in weight: (A)	—	37·0	—	52·3	—	53·2	62·5
(B)	16·9	20·0	33·6	45·1	47·4	48·6	—
(C)	20·2	—	43·4	50·1	59·7	60·8	61·4

Average maximum digestion in experiments (8) lasting 6 days or more was 53·0%.
(A) = best digestion series; (B) = worst digestion series; (C) = straw finely ground.

Digestion of the oat straw did not appear to be noticeably more rapid than that of wheat straw, nor did fine grinding apparently accelerate digestion. The slow rate of digestion of both straws indicated that in the animal rumen digestion would never proceed to completion. This was borne out by the following experiment in which the undigested residues (separated by washing on muslin) from the stomachs and faeces of sheep fed on oat and wheat straws as principal roughage were redigested by the silk bag technique in a sheep's rumen. The degree to which the residues were further digested is a measure of the inefficiency of the normal digestive processes. This was greater in the case of wheat straw than oat straw; however, the difference may be of little importance since the oat straw was not fed to the same sheep as the wheat straw.

Table 2. *Digestibility* in vivo *of stomach and faecal residues of sheep on diet containing straw as principal roughage*

	Material subjected to redigestion			
	Straw	Rumen residue	Abomasum residue	Faecal residue
% loss in weight of oat straw and residues 5 days redigested	52	18	20	12
% loss in weight of oat straw and residues 10 days redigested	64	24	26	16
% loss in weight of wheat straw and residues 7 days redigested	—	37	—	40
	—	38	—	31
	—	36	—	33

Digestion of cellulose

Table 3. *Digestion of prepared cellulose* (*0·5 g. portions*)

	Period of digestion, days				
	1	2	3	4	6
% digestion of filter paper *in vitro*	—	56	—	100	—
% digestion of 'native' cellulose from oat straw *in vitro*	—	58	—	87	91
% digestion of filter paper *in vivo*	38	—	78	—	—
	26	64	93	—	—

Table 4. *Digestion of native cellulose in straws*, in vivo

Native cellulose in: wheat straw = 50 %; oat straw = 48 %. 0·5 g. samples of straw digested.

	Period of digestion, days					
	1	2	3	4	5	7
% loss of native cellulose in wheat straw	20	36	36	52	52	68
% loss of native cellulose in oat straw	25	33	50	62	54	—

Comparison of the rates of digestion of isolated cellulose with the rate of digestion of cellulose in straw indicates that about 25 % of the cellulose in straw is in a condition similar to that of isolated cellulose, since the first day's cellulose digestion in straw does not fall far short of that of isolated cellulose; after this the rate of digestion of cellulose in straw falls off considerably and 30–40 % of cellulose remains undigested even after a week's digestion.

The cellulose contents of the materials in the experiment recorded in Table 2 furnish the data for Table 5.

Table 5. *Digestibility of native cellulose in stomach and faecal residues of sheep on a diet containing oat straw as principle roughage*

Native cellulose in: oat straw = 46 %; rumen residue = 40 %; abomasum residue = 42 %; faecal residue = 34 %.

	Material subjected to redigestion			
	Straw	Rumen residue	Abomasum residue	Faecal residue
% loss in weight of native cellulose in straw and residues 5 days redigested	61	25	24	12
% loss in weight of native cellulose in straw and residues 10 days redigested	74	30	33	12

Contrary to the findings of Trautmann & Asher [1939], the lower percentage of cellulose in faecal residue than in residue from the stomachs and the greater completeness of cellulose digestion in the faecal material, as measured by the degree to which redigestion is possible, indicate that cellulose digestion does occur in the gut at a lower level than the abomasum. However, it will be seen that cellulose digestion is far from complete in the animal. Not only is further cellulose digestion possible if the period of action of the flora of the rumen is prolonged, but a hard core of indigestible cellulose remains after normal digestion in the animal's whole gut followed by 10 days' rumen digestion in a silk bag. This hard core of cellulose constitutes 35 % of the residual material.

Digestion of pentosan-polyuronide fractions

The literature indicates that these substances, often referred to as hemicelluloses or simply as pentosans, are readily fermented. König *et al.* [1907], Honcamp & Baumann [1921], Honcamp *et al.* [1921] and Fraps [1930] report considerable disappearance of pentosans during passage through the ruminant. Sitschev [1940; 1941] obtained almost 100 % utilization of *d*-xylose by ruminants. Norman [1929] and Acharya [1935] found that hemicellulose disappeared before cellulose in anaerobic fermentations of straw when no external inoculum was added.

Wheat straw hemicellulose was obtained by heating straw with 1·5 % KOH for 24 hr. on a water bath, acidifying the filtered extract with acetic acid and precipitating the crude hemicellulose with alcohol. The precipitate was taken up with *N*/2 KOH and measured out into 10 ml. aliquot portions for fermentation. Each was then neutralized to phenolphthalein with acetic acid. To these and to controls containing an equivalent amount of potassium acetate, 10 ml. of fresh muslin filtrate of rumen ingesta were added.

After fermentation for the periods stated, conc. H_2SO_4 was added to the experimental and control materials to make a final concentration of 2·5 % H_2SO_4; both were then heated on the water bath for 15 hr. [cf. Weihe & Phillips, 1940]. The hydrolysates were filtered and the sugar determined in the filtrates by Bertrand's method. The copper reduction in the controls was subtracted from that in the experiments and from the results the hemicellulose remaining at progressive stages of digestion was calculated in terms of xylose since this would be the principal sugar in the hydrolysate.

Table 6. *Digestion of prepared hemicellulose* in vitro

Solution A contained 50 mg. of hemicellulose per 10 ml. calculated as xylose.
Solution B contained 42 mg. of hemicellulose per 10 ml. calculated as xylose.

	Period of digestion, hr.				
	2	4	5	6	24
% digestion of hemicellulose (as xylose) in solution A	—	—	43	—	101
% digestion of hemicellulose (as xylose) in solution B	12	15	—	35	87

Digestion of furfuraldehyde-yielding material in wheat straw, calculated as xylan during a period of 7 days *in vivo* amounted to 52 % for the total material and 60 % for xylan in native cellulose.

Since the pentosan-polyuronide fraction of straw is probably not homogeneous it is possible that the readily fermented material concerned in Table 6 represents only a part of the whole. It is plain, however, that even if all this fraction is as rapidly fermented in the isolated state, in straw its digestion is checked, as was the case with cellulose, since some 40–50 % of the fraction remained undigested after 7 days' digestion *in vivo*.

Digestion of lignin

The literature contains few references to the digestibility of lignin by ruminants. König *et al.* [1907] report 28 % digestion of lignin in pea straw and hays. Honcamp & Baumann [1921] and Honcamp *et al.* [1921] found very slight digestion of lignin in straw but better digestion in hays. Csonka *et al.* [1929] fed lignin, isolated by alkali extraction, to dogs and to a heifer and measured the loss of methoxyl groups, presumably derived solely from lignin, and the excretion of benzoic acid derived from the aromatic nuclei in lignin. Both methods showed a slight degree of utilization of lignin by the heifer. Hale *et al.* [1940] made lignin the basis from which they calculated digestibility coefficients of other fodder constituents. From the results obtained they deduced that the assumption on which the calculation of the coefficients was based, that is, that lignin is not digested in the rumen, was true.

Table 7. *Digestion of lignin in straw (0·5 g. samples of straw digested)*

Lignin in: oat straw = 16·6 %; wheat straw = 22·4 %.

	Period of digestion, days			
	2	4	7	9
% digestion of lignin in oat straw *in vitro*	6	12	20	20
% digestion of lignin in wheat straw *in vivo*	—	—	31	—

Table 7 shows a definite digestion of lignin in straws. The percentage potential digestion is not, however, so great as is the case with cellulose and hemicellulose; it is therefore possible that digestion in the animal is too small to be measurable in digestibility trials. However, lignin cannot be regarded as wholly indigestible.

Effect of alkali pretreatment on digestion of straw

It has been shown in preceding sections that the digestion of isolated cellulose and pentosan-polyuronides is subject to a retarding effect when these materials are in their natural state in the straw. This effect is generally supposed to be due to an association between two or more of the straw constituents. Considerable controversy exists as to the nature of this association, the volume of evidence and theory being much increased by the importance of the problem in the wood pulp industry. Briefly, the theories held are as follows:

(*a*) That cellulose and lignin are very intimately associated but not chemically combined.

(*b*) That cellulose and lignin are chemically combined.

(*c*) That hemicellulose and lignin are chemically combined.

Any reagent which breaks the association of any two of these constituents would necessarily promote the digestion not only of those constituents but also indirectly of the third, by clearing the way for the approach of fermentative bacteria. In the course of a long series of experiments mainly by German workers, soaking in cold dilute alkali has been shown to be sufficient to render cellulose in straw notably more digestible without producing any great loss in the organic constituents of the straw.

The straw pulp used in the experiments recorded below was obtained by soaking the straw in 1·5% NaOH or KOH for 24 hr. then washing the resulting pulp with cold water until free from alkali.

The digestions recorded in Tables 8 and 9 were all performed by the *in vivo* method. For those in Table 9, 5 g. of straw or pulp were suspended in several silk bags for a week in a sheep's rumen. The residues were mixed and analysed. It was found that one large bag containing the whole of the sample was very difficult to retrieve through the fistula at the end of the digestion.

The results listed in Table 8 indicate that the alkali pulp is a very much more digestible material than the straw from which it is derived. Taking into account the limited intake of food of the animal, the pulp supplies very much more digestible material for a given

Table 8. *Effect of alkali pretreatment on wheat straw digestibility* in vivo

100 g. straw gave 72 g. pulp with 1·5% NaOH. Period of digestion was 7 days.

% loss of weight of ...	(*a*) Straw	(*b*) Pulp	(*c*) Pulp calculated on basis of straw = 100%
	55·5	85·6	62·0
	53·8	80·5	58·5
	51·4	77·9	56·3
	54·7	82·2	59·4
Average	53·8	81·5	59·0

Rate of digestion of wheat straw and wheat straw pulp

	Period of digestion, days				
	1	2	3	4	7
% loss in weight of straw	11·8	26·8	36·9	34·7	—
	15·9	29·5	32·7	—	50·5
	20·5	29·4	35·8	34·5	—
Average	16·1	28·6	35·1	34·6	50·5
% loss in weight of straw pulp	13·7	23·3	37·2	41·1	—
	16·0	31·1	48·8	—	88·2
	27·2	30·8	47·9	62·9	—
Average	19·0	28·4	44·6	52·0	88·2

intake than does the corresponding amount of straw (compare columns (*a*) and (*b*), Table 8). If, however, the yields of digestible material from straw by feeding directly or by pulping and then feeding are compared, it will be seen that the latter process yields a somewhat greater percentage upon the straw weight taken (compare columns (*a*) and (*c*), Table 8).

Table 9 shows the effect of pulping on the digestibility of straw constituents. It has generally been assumed that cellulose is the chief constituent affected by the pulping

Table 9. *Effect of alkali pretreatment on digestion of wheat straw constituents* in vivo (*pulp obtained with 1·5 % KOH*)

	Material analysed			
	Straw per 93·2 g. dry wt. from 100 g. straw	Straw digest per 46·4 g. obtained from 100 g. straw	Pulp per 82·0 g. obtained from 100 g. straw	Pulp digest per 16·4 g. obtained from 100 g. straw
Total pentosan-polyuronide, as g. xylan	27·7	13·4	24·9	3·9
True cellulose, g.	38·7	15·5	39·3	4·4
Xylan in native cellulose, g.	14·8	5·9	16·3	2·2
Lignin, g.	22·4	15·4	20·1	5·9

process. It will be seen from this table, however, that the digestibility of the pentosan-polyuronide fraction, both xylan in cellulose and hemicellulose not associated with cellulose, is notably increased. An interesting and unexpected result is also the marked effect of the alkali pretreatment on the digestibility of lignin. Thus, whatever may be the nature of the linkage, chemical or mechanical, of straw constituents which is broken down by the alkali, the third principal constituent is apparently not unaffected. The value of the process is generally ascribed to its effect upon the digestibility of cellulose. However, the ease of fermentation and relatively high content in straw of the pentosan polyuronide fraction give added importance to a process which enhances the digestibility of this fraction also.

Discussion

If the reasons for wastage of straw constituents during the ruminant's digestive process be examined, it will be seen that innate indigestibility of the isolated materials, even in the case of lignin, is not responsible for the wastage. However, a form of association between constituents exists which results in a hard core, containing a proportion of all the main constituents, which is not digestible even by prolonged exposure to rumen bacteria. Over and above the materials wasted in this way, too rapid a passage through the alimentary tract results in wastage of material which, if subjected to more prolonged action by rumen bacteria, would be digested. The digestibility of faecal residue on re-digestion provides evidence of this.

Alkali pretreatment results in a much greater potential digestibility of straw constituents, due to the presumed breaking of some link between them. However, the rate of digestion in the early stages (first 2 days) is not notably greater in pulped straw. Thus wastage through too rapid a passage out of the rumen may not be avoided by the alkali pretreatment. Evidence for this is given by the fact that the faecal residue of a sheep fed on alkali pulp was still 30 % digestible as the result of being suspended for a week in a sheep's rumen. It would thus seem that, in order to achieve maximum utilization of straw nutrients, some form of predigestion *in vitro* might be of use. It is possible by anaerobic fermentation of straw with cultures from the rumen to obtain large quantities of volatile acids. The usefulness of such a method cannot be judged until more evidence is available as to the importance of the volatile acids in ruminant nutrition. This problem is now being investigated by us (Animal Physiology unit).

Bacterial predigestion might further be of value if it were possible to achieve considerable synthesis of protein from inorganic nitrogen with straw as a source of carbon. Experiments with this end in view were, however, disappointing. While uptake of ammonium salts when cellulose or starch were digested was considerable, no uptake of ammonium salts when straw was the substrate could be demonstrated.

Summary

1. Experiments are quoted showing the rate of digestion and digestibility of whole straw in the sheep's rumen, and the degree to which straw remains undigested during its passage through the alimentary canal of a sheep. It is concluded that much potentially digestible material is wasted.

2. Cellulose is slowly but completely digested in the isolated state, but in straw, while digestion proceeds at first at a rate comparable with that of isolated cellulose, it slows down leaving a substantial proportion of cellulose undigested.

3. Isolated hemicellulose is wholly digested, while in straw the pentosan-polyuronide fraction is only partially digested.

4. Loss of lignin occurs during digestion of straw in the rumen or with rumen contents *in vitro*.

5. Alkali pretreatment of straw produces a pulp which is much more digestible than the original straw. The digestibility of pentosan-polyuronide and of lignin, as well as of cellulose, is favourably affected.

6. The reasons for wastage of nutrients in straw fed to ruminants and the means by which the wastage can be avoided are discussed.

I wish to thank Prof. D. M. S. Watson who suggested to me the problem of predigestion of straw with cultures from the rumen, Dr A. T. Phillipson who kindly undertook some of the *in vivo* digestions using a sheep at the Institute of Animal Pathology, Cambridge, and Mr E. Turner who gave valuable technical assistance.

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46. The Phosphatides of *Hevea Brasiliensis*

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The presence of phosphatide in *Hevea* latex had long been suspected but it was not until 1931 that Rhodes & Bishop isolated phosphatidic material containing 0·59 % N and 1·72 % P from the serum of latex which had been coagulated with ethanol. The crude material of these authors represented 0·2 % of the latex, and on treatment with acetone yielded an insoluble fraction containing 0·88 % N and 2·56 % P (40 % of the former product). This latter fraction was, according to the accepted analytical composition of phosphatide, still very impure. The work of Rhodes & Bishop was repeated by Altman & Kraay [1940] who isolated in very small amounts a product giving much the same analyses, and extended the findings of Rhodes & Bishop by showing that the crude phosphatide contained both choline and glycerophosphoric acid.

It is very probable that the phosphatide, although present in such small amount, is one of the important non-rubber constituents of latex. Judging from the ease with which phosphatides emulsify in aqueous media, there seems little doubt that the phosphatidic material assists in the maintenance of latex as a stable emulsion. It has been suggested [Bollman, 1926] that crude phosphatide preparations act as the natural antioxidants in vegetable oils. This suggestion has been disputed by Diemair & Fox [1939] and other workers who state that this property is not possessed by pure phosphatide although the complex system from which the phosphatide had been separated was an active antioxidant. Diemair *et al.* [1940] have advanced the opinion that the active antioxidant is a protein-phosphatide complex.

The present work was undertaken as part of an investigation of the non-rubber constituents of latex. The crude phosphatide was prepared by the method of Rhodes & Bishop [1931], the preliminary treatment being carried out at the Rubber Research Laboratories, Ceylon, under the direction of Mr O'Brien to whom the author's thanks are due. The raw material was treated by the method outlined below. The crude phosphatide was fractionated by a method which was essentially that used by Channon & Foster [1934] in their work on the phosphatides of wheat germ. The fractionation resulted in the isolation of a fairly pure form of phosphatidic acid (the possible source of which is discussed later) and an impure form of lecithin. The lecithin fraction contained little or no kephalin (only about 5·5 % of the N in this fraction was amino-N), a finding which was unexpected.

Experimental

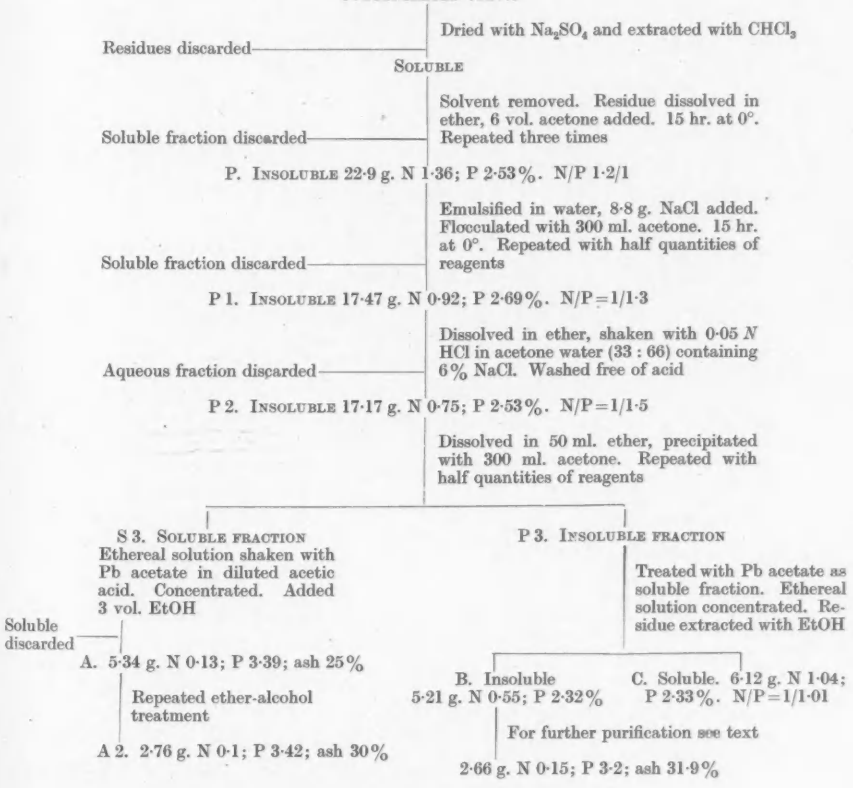
Preparation of the crude material

Freshly tapped latex was coagulated by pouring into 3 vol. of 95 % ethanol. The serum was concentrated *in vacuo* at 40° and the concentrate sealed *in vacuo* and shipped to England. Three samples representing 19·5 l. latex were received in good condition. The residues were mixed with anhydrous Na_2SO_4 and extracted with $CHCl_3$. The extract was concentrated *in vacuo*, dissolved in ether and the phosphatide precipitated by the addition of 6 vol. of acetone. After 15 hr. at 0° the supernatant liquid was decanted and the residue again treated with ether and acetone. This precipitation was repeated three times. 22·95 g. of material containing 1·36 % N and 2·53 % P were isolated.

Fractionation of the phosphatide

Plant phosphatides have been shown by several workers [Smith & Chibnall, 1932, 2; Channon & Foster, 1934; and Jordan & Chibnall, 1933] to contain lecithin and kephalin with varying amounts of phosphatidic acid, the latter compound being present as a salt of Ca, Mg or K (some workers state that the Na salt exists in the plant). It was considered likely that the phosphatide of *Hevea* might be a similar mixture and for this reason the fractionation procedure was essentially that of Channon & Foster [1934]. These workers have described the method in great detail and the accompanying diagram will suffice to illustrate the course of the fractionation.

Diagram 1.


CONCENTRATED SERUM
Dried with Na_2SO_4 and extracted with $CHCl_3$
Residues discarded
SOLUBLE
Solvent removed. Residue dissolved in ether, 6 vol. acetone added. 15 hr. at 0°. Repeated three times
Soluble fraction discarded
P. INSOLUBLE 22·9 g. N 1·36; P 2·53%. N/P 1·2/1
Emulsified in water, 8·8 g. NaCl added. Flocculated with 300 ml. acetone. 15 hr. at 0°. Repeated with half quantities of reagents
Soluble fraction discarded
P 1. INSOLUBLE 17·47 g. N 0·92; P 2·69%. N/P=1/1·3
Dissolved in ether, shaken with 0·05 N HCl in acetone water (33 : 66) containing 6% NaCl. Washed free of acid
Aqueous fraction discarded
P 2. INSOLUBLE 17·17 g. N 0·75; P 2·53%. N/P=1/1·5
Dissolved in 50 ml. ether, precipitated with 300 ml. acetone. Repeated with half quantities of reagents
S 3. SOLUBLE FRACTION
Ethereal solution shaken with Pb acetate in diluted acetic acid. Concentrated. Added 3 vol. EtOH
P 3. INSOLUBLE FRACTION
Treated with Pb acetate as soluble fraction. Ethereal solution concentrated. Residue extracted with EtOH
Soluble discarded
A. 5·34 g. N 0·13; P 3·39; ash 25%
Repeated ether-alcohol treatment
A 2. 2·76 g. N 0·1; P 3·42; ash 30%
B. Insoluble 5·21 g. N 0·55; P 2·32%
C. Soluble. 6·12 g. N 1·04; P 2·33%. N/P=1/1·01
For further purification see text
2·66 g. N 0·15; P 3·2; ash 31·9%

Results

Fraction A 2. This represents a reasonably pure form of lead phosphatidate. Micro-analysis gave C 50·6; H 8·3; P 3·42; ash 30·0 (Smith & Chibnall give C 51·2; H 7·7; P 3·48; ash 33 for lead phosphatidate but the analysis will obviously depend on the nature of the fatty acids present). On hydrolysis it yielded 60·4% fatty acids (I.V. 112; mol. wt. 290·5). A Twitchell lead soap separation of the latter indicated the presence of 21·6%

saturated acids (mol. wt. 280) and 79·4 % liquid acids (I.V. 139; mol. wt. 303). Dihydroxystearic acid M.P. 130° and tetrahydroxystearic acid M.P. 156–157° were obtained on oxidation of the unsaturated fraction by the method of Haworth [1929].

Fraction B. When isolated this fraction contained 0·55 % N and 2·35 % P. Extraction with alcohol, or precipitation with alcohol from ethereal solution, did not increase the purity of the fraction; lead was therefore removed and the free acid emulsified in water, precipitated with acetone and the lead salt regenerated. Severe loss occurred during this procedure but 2·66 g. of material were isolated which contained 0·15 % N; 3·15 % P; ash 31·9 %; 49·15 % C; 7·4 % H. On hydrolysis it yielded 60 % of fatty acids (I.V. 90; mol. wt. 326) of which acids 26 % were solid acids (mol. wt. 326) and 74 % liquid acids (I.V. 123; mol. wt. 327). It thus represented a further amount of fairly pure lead phosphatidate. It differed in fatty acid composition from A 2.

Fraction C. This fraction is a crude lecithin and it was impossible to effect any further purification. The difficulties of purifying small samples of phosphatide have been stressed by Smith & Chibnall [1932, 2] and Jordan & Chibnall [1933]. More recently Diemair *et al.* [1937] have isolated phosphatides from oats, barley and wheat which contained only 1·0 % N and 2·2 % P although they were apparently free from sugar. Working & Andrews [1941] state 'It is difficult to imagine what impurities might cling so firmly as not to be removed by the methods used successfully with materials of animal origin'. The results obtained by Rhodes & Bishop [1931] and Altman & Kraay [1940] indicate that they too found difficulty in purifying their products beyond the limits of N 1·0 %, P 2·5 %.

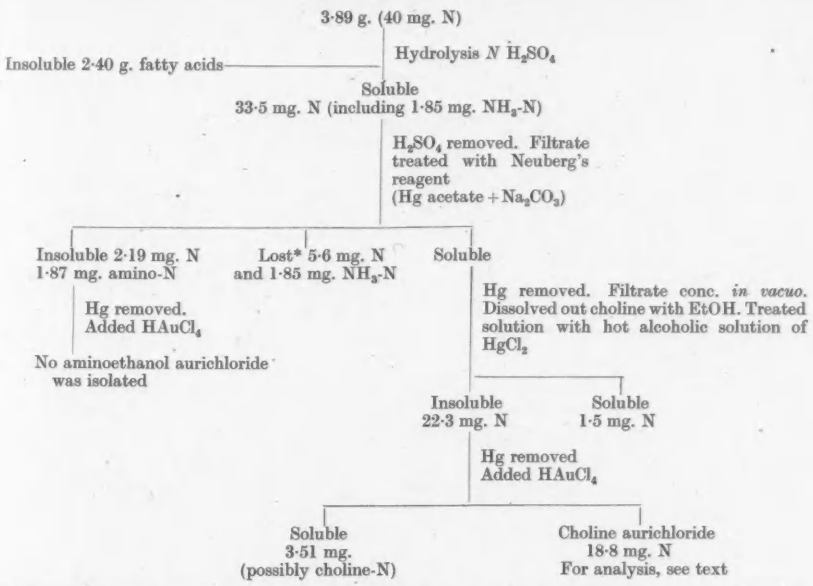
Analysis of fraction C. 3·89 g. were hydrolysed with *N* H_2SO_4 for 8 hr. 2·40 g. of fatty acids were recovered (62 % yield), I.V. 111; mol. wt. 315. 2·01 g. were fractionated (Twitchell) into 25 % saturated acids (mol. wt. 310) and 75 % unsaturated acids (I.V. 150; mol. wt. 326). Dihydroxystearic (M.P. 125°) and tetrahydroxystearic acid (M.P. 158°) were obtained by oxidation of the unsaturated fraction.

The aqueous fraction was concentrated *in vacuo* and analysed for bases and reducing sugar. Reducing sugar was determined by the method of Maclean [1919] and some 3·3 % was found (calculated as glucose). This figure is a minimal one since appreciable quantities of carbohydrate are decomposed during the acid hydrolysis [cf. Smith & Chibnall, 2]. Even if this is so, a large proportion of the impurity remains uncharacterized [cf. Diemair *et al.* 1937]. The total N of the aqueous fraction was 33·5 mg., some 6·5 mg. (16 %) being lost during hydrolysis. Similar losses (22 %) were reported by Smith & Chibnall [1932, 2] and by Jordan & Chibnall [1933], who found that only 65 % of the original N passed into the aqueous filtrate. It is possible that the loss of N is coupled with the lower amount of reducing sugar (q.v.) in the formation of an insoluble humin. Amino-N as determined by the method of Van Slyke amounted to approximately 1·85 mg., which, if it were all aminoethanol-N, would correspond to 8·15 mg. aminoethanol or to about 98 mg. kephalin. (Thus the kephalin content of the fraction cannot be more than 2·5 % of the crude phosphatide or about 4 % of the calculated phosphatide.)

Isolation of the bases. The aqueous filtrate was freed from H_2SO_4 and analysed for bases. The method has been described in detail by Smith & Chibnall [1932, 2] and depends on the precipitation of primary amines (including amino-acids) by the reagent of Neuberg & Kerb [1912]. Choline is soluble in the reagent but is precipitated almost quantitatively by $HgCl_2$. The following scheme indicates the course of the separation and the distribution of N.

The choline aurichloride was isolated in two crops: (1) M.P. 268–270°; Au 44·14; C 13·65; H 3·2; (2) M.P. 265°; Au 44·5; C 13·6; H 3·2. (The analysis in crop 2 was corrected for a small amount of free gold which was found to be contaminating the sample.) $C_5H_{14}ON.AuCl_4$ has M.P. 269–270° and requires Au 44·5; C 13·5; H 3·2 %.

Diagram 2.


3·89 g. (40 mg. N)
Insoluble 2·40 g. fatty acids
Hydrolysis N H_2SO_4
Soluble
33·5 mg. N (including 1·85 mg. NH_3-N)
H_2SO_4 removed. Filtrate treated with Neuberg's reagent (Hg acetate + Na_2CO_3)
Insoluble 2·19 mg. N
1·87 mg. amino-N
Lost* 5·6 mg. N and 1·85 mg. NH_3-N
Soluble
Hg removed. Added $HAuCl_4$
No aminoethanol aurichloride was isolated
Hg removed. Filtrate conc. *in vacuo*. Dissolved out choline with EtOH. Treated solution with hot alcoholic solution of $HgCl_2$
Insoluble 22·3 mg. N
Soluble 1·5 mg. N
Hg removed Added $HAuCl_4$
Soluble 3·51 mg. (possibly choline-N)
Choline aurichloride 18·8 mg. N For analysis, see text

* The loss of 5·6 mg. N was due largely to the difficulty of washing the large precipitates of HgS. The 1·85 mg. ammonia-N would also be lost during the concentration of the alkaline Neuberg filtrate.

Discussion

From 19·5 l. latex 22·9 g. of crude material (N 1·36; P 2·53) were isolated. This material, on treatment with water and acetone yielded 17·5 g. (N 0·92; P 2·69) which is regarded as the true starting material. It represents 0·09 % of the latex. From this material were isolated 6·12 g. lecithin (N 1·04; P 2·33) and 5·42 g. lead phosphatidate (P 3·2–3·4; ash 30–32; N *ca.* 0·1). The yield of crude lecithin (60–65 % pure) is approximately 35 % of the initial material (i.e. 0·03 % latex) and the yield of phosphatidic acid is equivalent to 24 % of the starting material (i.e. 0·02 % latex). The loss of lead phosphatidate was large during the final stages of purification and the amount of phosphatidic acid might well be about 6·0 g. (35 % of fraction P 1).

As stated above the lecithin fraction was found to contain little or no kephalin. This result was surprising and it seemed possible that at least some of the phosphatidic acid might be a secondary product derived from the breakdown of kephalin during the concentration of the alcoholic serum.

Although some of the phosphatidic acid may be derived from kephalin breakdown the presence of phosphatidic material in other plants (Brassicae, Graminae, Leguminosae etc.) suggests that one might expect to find the acid as a natural constituent of fresh latex.

Evidence has been obtained which suggests that phosphatidic acid does occur in fresh latex. Thus the Ca content of P 1 was 0·51 % and that of P 2 about 0·1 %. (Neither fraction contained Mg.) The Ca content of P 1, if all the phosphatidic acid isolated (4·2 g.) were present as the Ca salt, would be about 1·2 % whilst that of P 2 should be negligible. The Ca found suggests that 40 % of the phosphatidic acid in P 1 is present as Ca phosphatidate. If any free phosphatidic acid were present in the crude phosphatide (P) one might expect to lose a good deal of it during the ether-acetone fractionation and

the presence of phosphatidic acid after this stage of the fractionation suggests that it is present in salt form in P 1. Unfortunately it was not possible to analyse P 1 for the alkali metals (Na and K) since NaCl had been used to assist in the flocculation of P 1 with acetone. In order to check this point, and the apparent absence of kephalin, a sample of phosphatide was prepared from dried latex (a product in which the breakdown should have been small).

1·48 g. of crude phosphatide were obtained (N 1·2 %, P 2·2 %) from 850 g. dried latex. The crude material after three precipitations with acetone was dissolved in ether and the solution shaken with 0·05 *N* HCl in 40 % acetone-water. The aqueous extract was analysed for Na, K, Ca and Mg. Na and Mg were absent. The extract was found to contain the equivalent of 0·48 % Ca (estimated by the method of Wiley [1931]) and 2·6 % K (estimated by the method of Hamid [1926]).

If these elements were in combination with phosphatidic acid then the crude phosphatide would contain 0·14 g. (9·4 %) calcium phosphatidate and 0·41 g. (27·6 %) dipotassium phosphatidate. Thus fraction P 1 (diagram 1) should contain 5·9 g. phosphatidic acid (0·03 % latex). Only 4·2 g. (0·02 % latex) were isolated, since severe losses occurred during the final purification of the phosphatidic acid fractions A 2 and B. It is considered therefore that the total amount of phosphatidic acid present in fraction P 1 may well be 5·9 g. (0·03 % latex) and that it is probably present as both the calcium and potassium salts in the ratio of 1 : 3.

No amino-nitrogen, in amounts greater than those suggested above, could be detected in the phosphatide and it must be concluded that the phosphatide of *Hevea Brasiliensis* does not contain appreciable amounts of kephalin. This finding is in keeping with that of Altman & Kraay [1940].

The fatty acids of the fractions A, B and C had I.V. 90–112 and contained 21–26 % saturated acids, whilst the fatty acids from the glycerides [Tristram, 1942] had I.V. 135–148 and contained 24–26 % saturated acids. Thus the glyceride fatty acids are slightly more unsaturated than the phosphatide fatty acids. This finding is similar to those of Smith & Chibnall [1932, 1, 2] who found that the glyceride and phosphatide fatty acids of cocksfoot grass (*Dactylis glomerata*) had I.V. 170 and 128–144 respectively.

In a recent paper [Baker, 1941] it was stated that on purifying latex by creaming or centrifuging the nitrogen content of the rubber was reduced to a lower limit of 0·03 %. This N was said to be due to the 'phospholipin-sterol complex which is also thought to be associated with the rubber phase'. Now if the pure phosphatide of *Hevea Brasiliensis* is assumed to be lecithin only, the residual N referred to by Baker corresponds to the presence in the crude rubber of 1·7 % of lecithin. If there is any phosphatidic acid present the amount of phosphatide would be even higher (1·7–3·4 %). Now the maximum amount of phosphatide (even in the crude condition) which has been isolated is 0·2 % (N 0·59; P 1·72) [Rhodes & Bishop, 1931] and there is every reason to suppose that some of the N in this product is non-phosphatide-N. The assumption of Baker could be tested by a determination of the phosphorus content (0·06 if the phosphatide is lecithin only). The statement of Baker infers that the phosphatide is only removed from the rubber globule with great difficulty. It must be pointed out however that the lipin was isolated from the serum after coagulation of the latex with alcohol. The fact that crepe rubber contains little or no phosphatide may indicate that the reverse is the case, namely that the phosphatide, whilst assisting in the maintenance of the latex in equilibrium, will appear in the aqueous fraction when that equilibrium is upset.

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